

**Transplantation of Hodgkin's and  
Non-Hodgkin's Lymphomas into SCID Mice**

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**Presented to the University of Edinburgh for the  
Degree of Doctor of Philosophy  
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To my mum, Dorothy and in memory of my dad, Rodger



## **Declaration**

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Jacqueline A. Lowrey

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## **Abbreviations**

<b>ABC</b>	Avidin Biotin Complex
<b>AIDS</b>	Acquired Immunodeficiency Syndrome
<b>BCIP</b>	5-Bromo-4-Chloro-3-Indoxyl Phosphate
<b>BL</b>	Burkitt's Lymphoma
<b>BSA</b>	Bovine Serum Albumin
<b>Cb</b>	Centroblastic
<b>Cb Cc Foll</b>	Centroblastic Centrocytic Follicular
<b>CMV</b>	Cytomegalovirus
<b>DAB</b>	3,3'-Diaminobenzidine
<b>DEPC</b>	Diethylpyrocarbonate
<b>DNA</b>	Deoxyribonucleic Acid
<b>EBER</b>	Epstein-Barr Virus Encoded RNAs
<b>EBNA</b>	Epstein-Barr Nuclear Antigen
<b>EBV</b>	Epstein-Barr Virus
<b>ELISA</b>	Enzyme Linked Immuno-Sorbent Assay
<b>EMA</b>	Epithelial Membrane Antigen
<b>FCS</b>	Foetal Calf Serum
<b>FITC</b>	Fluorescein Isothiocyanate
<b>Glut</b>	Glutamine
<b>GM CSF</b>	Granulocyte Monocyte Colony Stimulating Factor
<b>HD</b>	Hodgkin's Disease
<b>H&amp;E</b>	Haematoxylin & Eosin
<b>HHV</b>	Human Herpesvirus
<b>HOME</b>	Highly Optimised Microscope Environment
<b>HTLV</b>	Human T Cell Lymphotropic Virus
<b>ICAM</b>	Inter-cellular Adhesion Molecule
<b>Ig</b>	Immunoglobulin
<b>IL</b>	Interleukin
<b>Immb</b>	Immunoblastic
<b>i.p.</b>	intraperitoneal
<b>LAK</b>	Lymphokine Activated Killer
<b>L&amp;H</b>	Lymphocytic & Histiocytic
<b>LCA</b>	Leucocyte Common Antigen
<b>LCAL</b>	Large Cell Anaplastic Lymphoma

<b>LCL</b>	Lymphoblastoid Cell Line
<b>LDHD</b>	Lymphocyte Depleted Hodgkin's Disease
<b>LFA</b>	Lymphocyte Function Associated
<b>LMP</b>	Latent Membrane Protein
<b>LPD</b>	Lymphoproliferative Disease
<b>LPHD</b>	Lymphocyte Predominant Hodgkin's Disease
<b>MCHD</b>	Mixed Cellularity Hodgkin's Disease
<b>ML</b>	Malignant Lymphoma
<b>mRNA</b>	messenger Ribonucleic Acid
<b>NBT</b>	Nitro Blue Tetrazolium
<b>NHL</b>	Non-Hodgkin's Lymphoma
<b>NK</b>	Natural Killer
<b>NPC</b>	Nasopharygeal Carcinoma
<b>NSHD</b>	Nodular Sclerosis Hodgkin's Disease
<b>NSS</b>	Normal SCID Serum
<b>OD</b>	Optical Density
<b>OPD</b>	O-Phenylene Diamine
<b>PBL</b>	Peripheral Blood Lymphocytes
<b>PBS</b>	Phosphate Buffered Saline
<b>PCR</b>	Polymerase Chain Reaction
<b>Pen</b>	Penicillin
<b>PMT</b>	Photo Multiplying Tube
<b>REAL</b>	Revised European American Lymphoma
<b>RS/H</b>	Reed-Sternberg/Hodgkin
<b>s.c.</b>	subcutaneous
<b>SCID</b>	Severe Combined Immune Deficient
<b>Strep</b>	Streptomycin
<b>TBS</b>	Tris Buffered Saline
<b>TcR</b>	T cell Receptor
<b>Tespa</b>	3-aminopropyltriethoxysilane
<b>TGF</b>	Transforming Growth Factor
<b>TNF</b>	Tumour Necrosis Factor
<b>TR</b>	Terminal Repeat
<b>VDJ</b>	Variable Diversity Joining
<b>VIP</b>	Vacuum Impregnation Processor



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## **Abstract**

In an attempt to establish an *in vivo* animal model to study Hodgkin's disease (HD) and the histogenesis of the neoplastic cell of the disease - the Reed-Sternberg (RS) cell, and to study non-Hodgkin's lymphomas (NHL), severe combined immunodeficient (SCID) mice were transplanted with fresh biopsy material from 17 cases of HD and 25 cases of NHL. Five of the Hodgkin's (3 lymphocyte predominant, 1 nodular sclerosing and 1 mixed cellularity) and 7 of the non-Hodgkin's lymphomas (3 centroblastic, 1 immunoblastic, 1 follicular lymphoma and 2 biopsies from a case of B cell large cell anaplastic lymphoma) produced human high grade B cell lymphomas in the mice. All of the Hodgkin's disease derived SCID tumours expressed the Epstein-Barr virus (EBV) gene products EBER, EBNA-2 and LMP-1, even though only 1 of the original biopsies showed EBV positive RS cells. However, EBV clonality analysis showed that the EBV positive clone present in the original biopsy was not present in the SCID tumour. Morphologically, these tumours resembled polymorphous immunoblastic lymphomas, although in each case there were a few RS-like cells. The HD-derived SCID tumours did not retain the phenotype of the original biopsies, instead exhibiting an activated B cell phenotype with high expression of the CD23 and CD43 antigens. All HD-derived SCID tumours showed clonal Ig gene rearrangements. Further characterisation demonstrated that all of the HD-derived SCID tumours showed Ig secretion and contained the normal diploid DNA content. One SCID tumour derived from a follicular lymphoma showed similar morphology, phenotype, identical EBV gene expression and properties of Ig secretion and diploid DNA content as the HD-derived SCID tumours. Three centroblastic and 1 immunoblastic lymphoma were EBV negative. They all showed near identical morphology and B cell phenotype, and identical Ig gene rearrangements to that of the original biopsies. In addition, no Ig secretion was seen and DNA aneuploidy was exhibited. SCID tumours derived from the large cell anaplastic lymphoma showed 2 types of lymphoproliferation; firstly immunoblastic tumours showing EBER, EBNA-2 and LMP-1 expression, an activated B cell phenotype with Ig gene rearrangements different to those of the original biopsy, Ig secretion and diploid DNA content; secondly large cell anaplastic tumours retaining the

EBV (EBER only) and restricted antigenic phenotype of the original biopsy, and showing identical Ig gene rearrangements to those of the original biopsy, no Ig secretion and DNA aneuploidy.

# **Chapter One**

## **Introduction**

This introduction describes both Hodgkin's disease and the non-Hodgkin's lymphomas in some detail, including a general introduction to clinical features and pathology of the diseases.

The Hodgkin's disease section includes a description of the previous phenotypic and genotypic studies carried out in an attempt to define the histogenesis of the neoplastic cell of Hodgkin's disease - the Reed-Sternberg cell - and the problems encountered in these studies.

In the non-Hodgkin's lymphomas section, the various sub-types encountered in this thesis are described in detail.

The aetiology of Hodgkin's disease and non-Hodgkin's lymphoma is then discussed with special reference to the Epstein-Barr virus, described in more detail because of its involvement in the SCID tumours grown in this thesis.

The alternative models used to investigate Hodgkin's disease and non-Hodgkin's lymphoma, such as cell lines and nude mice, and the associated difficulties are discussed and finally the model that is used in this thesis to investigate Hodgkin's disease and non-Hodgkin's lymphoma - the SCID mouse - is described in some detail.

Malignant lymphomas are primary tumours of the lymphoreticular system, most if not all arising from lymphocytes. They all exhibit the ability to disseminate or metastasise and most, if untreated, will be fatal although they do vary greatly, some being capable of a fatal outcome in a few weeks or months (high grade) while others may take many years to do so (low grade).

Malignant lymphomas can arise in almost any organ due to the widespread nature of the lymphoreticular system, but most originate in the primary or secondary lymphoid organs especially the lymph nodes. Lymphomas also tend to spread to other parts of the lymphoreticular system - wide-spread lymphadenopathy and splenomegaly are thus common features (Lee & Bird 1992).

Lymphomas are divided into 2 main groups - Hodgkin's Lymphoma or Disease (HD) and the Non-Hodgkin's lymphomas (NHL).

## **1.1 Hodgkin's Disease**

### **1.1.1 Clinical Features**

This was first described by Thomas Hodgkin (1832), being pathologically characterised following descriptions of the histological marker cell by Sternberg (1898) and Reed (1902) and which now bears their names - the Reed-Sternberg cell.

Hodgkin's disease is one of the most common types of lymphoma, accounting for about 40% of all malignant lymphoma cases in Western Europe and the USA (Smithers 1973). It has a biphasic age incidence, peaking in early adult life and again in late middle age (MacMohen 1966).

Lymph node enlargement, involving the neck and mediastinal nodes, and less commonly the axillary, inguinal and intra-abdominal nodes is by far the most common presenting feature (Colby et al 1981, Mauch et al 1993). Only rarely does the disease arise extra-nodally. In some cases systemic symptoms such as low grade intermittent pyrexia, sweating, pruritus and weight loss are seen. There can be anaemia sometimes accompanied by neutrophilia or eosinophilia. In the majority of cases the disease soon spreads via the lymphatics to adjacent nodal groups and subsequently to distant nodes - the spleen may also be involved at an early stage. Only later are extra-nodal sites involved. This pattern of spread forms the basis of the Ann Arbor clinical staging system - this is the important factor in selecting treatment for HD (Carbone et al 1971).

Approximately 75% of all patients who present with Hodgkin's disease should be cured of their disease. Radiotherapy alone may be used to treat Stage I or II disease. Some cases of Stage I and II and most patients with advanced Hodgkin's disease (Stage III or IV) are treated with one of the standard programmes of combination chemotherapy (reviewed by Urba & Longo 1992, Devita & Hubbard 1993).

### **1.1.2 Histology**

The diagnosis of HD is carried out on a histological basis, and the histopathological criteria for the diagnosis and classification of HD is well established (Lukes & Butler 1966, Lukes et al 1966, Lukes 1971, Colby et al 1981, Bennett et al 1991).

Diagnosis of Hodgkin's disease depends on the presence of the Reed-Sternberg (RS) cell - usually regarded as the neoplastic component of the disease (Lukes 1971). The classical Reed-Sternberg cell measures between 20-30 $\mu$ m in diameter and contains either a bi-lobed nucleus or 2 nuclei. Each lobe or nucleus has a large, prominent nucleolus (3-5 $\mu$ m) which is usually eosinophilic. There is often a clear zone around the nucleolus with a rim of chromatin condensation around the nuclear membrane. The cells show abundant, usually eosinophilic cytoplasm. The mononuclear version of the RS cell is called the Hodgkin cell - this is morphologically similar to the RS cell, but because it is also similar to other lymphoid cells, it is not so important in the primary diagnosis of the disease (Lukes & Butler 1966).

The RS cells (and Hodgkin cells) (RS/H) must be accompanied by an appropriate stromal response before a specific diagnosis of Hodgkin's disease can be made (Lukes 1971). A typical lymph node affected by the disease will have distorted architecture. In most cases, the paracortical areas (the T cell areas) are primarily affected, although sometimes it is the cortical B cell areas. There is a polymorphic cellular infiltrate consisting of lymphocytes, neutrophils, eosinophils, histiocytes or macrophages, plasma cells and fibre forming cells. The patterns of this cellular response are very varied depending on the sub-type and the prognosis of the disease. The RS/H cells may only be present in small numbers, even in extensive disease. The lymphocytes present are believed to be the host immune response to the RS/H cells - they are mostly T cells (Hsu et al 1985) (except in the nodular Lymphocyte Predominance sub-type of Hodgkin's, where the background lymphocytes are mostly polyclonal B cells (Abdulaziz et al 1984)) and were found to be predominantly mature helper T cells (CD3+, CD4+,



CD45RO+)(Martin & Warnke 1984, Poppema 1989). Because they are found in such close proximity to the perimeter of the RS/H cells, there has been speculation that some kind of reaction is taking place between them (Stuart et al 1977). T cells in Hodgkin's disease have been shown to be well equipped for cell-cell interactions by the presence of high levels of LFA-1 and LFA-2. RS cells express high levels of LFA-3 - the ligand for LFA-2. It has also been demonstrated that 'lymphocyte rosetting' of T cells with the Hodgkin cell line L248 can be inhibited by antibodies against LFA-1, LFA-2 and LFA-3 (Sanders et al 1988).

The histological appearance of Hodgkin's disease (i.e. low numbers of RS/H cells against a back-ground of reactive cells) and clinical features of Hodgkin's disease could be explained by local cytokine release by either the malignant RS/H cells or by the lymphoid population infiltrating the involved tissues. Expression of several cytokines have been demonstrated in RS cells in biopsy material including IL-1 (Hsu & Zhao 1986, Xerri et al 1992), IL-3 (Merz et al 1991), IL-4 (Merz et al 1991), IL-5 (Samoszuk & Nansen 1990), IL-6 (Jucker et al 1991), IL-9 (Merz et al 1991), TGF- $\beta$  (Hsu et al 1993) and TNF- $\alpha$  (Kretschmer et al 1990).

In addition to the above cytokines, HD-derived cell lines have also been found to express IL-8 (Gruss et al 1992) and CSF(GM-CSF)(Burrichter et al 1983). This cytokine expression could explain the infiltration of eosinophils (IL-5), neutrophils, macrophages and lymphocytes (IL-1), particularly CD4 T lymphocytes (IL-9), and the fever (IL-1, TNF- $\alpha$ ) and depressed cellular immunity (TGF- $\beta$ ) involved with the disease.

Expression of IL-6 and the IL-6 receptor have both been found on RS/H cells and Jucker et al (1991) suggest that the IL-6, either by an



autocrine or paracrine (produced by the back-ground CD4+ T cells) loop, may stimulate the growth of the neoplastic RS/H cells.

### **1.1.3      Histological Sub-typing of Hodgkin's Disease**

The nature of the cellular back-ground in which RS cells are found forms the basis of the Rye classification which divides Hodgkin's disease into 4 main sub-groups :- Lymphocyte Predominant (LP), Nodular Sclerosing (NS), Mixed Cellularity (MC) and Lymphocyte Depleted (LD). Each sub-type contains a specific sub-type of the RS cell as well as the classical RS cells (Lukes & Butler 1966, Lukes et al 1966, Butler 1992) (Figures 1.1 & 1.2).

#### **Lymphocyte Predominant Hodgkin's Disease**

This sub-type is now widely regarded as distinct from the other sub-types as a B cell lymphoma. It represents about 7% of cases in most series. It is only rarely encountered over the age of 40 and it shows a marked male predominance. This sub-type most commonly presents in the neck nodes, mediastinal involvement is rare (Mauch et al 1993).

There are 2 sub-types - Nodular and Diffuse (Lukes & Butler 1966, Lukes et al 1966). The affected lymph node is characterised by proliferation of mostly lymphocytes and some histiocytes (plasma cells and eosinophils are rarely found), and by the presence of the Lymphocytic and Histiocytic (L and H) variant of the RS cell. This cell, also known as the 'popcorn' cell, tends to be slightly smaller than the other RS cell variants (Figure 1.1). The nucleus shows lobation with multiple small nucleoli and the cytoplasm is pale and seldom abundant. These L and H cells are more abundant in the

Nodular sub-type where they are confined to large, poorly defined nodules in which they often form small aggregates (Colby et al 1981). Other enlarged follicles showing features of a nodal lesion known as progressive transformation of germinal centres with or without L and H cells may also be present (Hansmann et al 1990). Classical RS cells are rarely found in this LP sub-type (Butler 1992), indeed if they are too readily found the sub-typing should be reconsidered as cellular phase NSHD or MCHD. However the presence of the L and H cell is essential in diagnosis of LPHD and to prevent mis-diagnosis as progressive transformation of germinal centres.

### Nodular Sclerosis Hodgkin's Disease

This sub-type is the most common form of Hodgkin's disease (about 65%). It mainly affects young adults and is the predominant sub-type in young females. This sub-type presents most commonly in the neck nodes and mediastinum (Colby et al 1981, Mauch et al 1993).

In addition to classical RS cells, the lacunar variant is also found. Lacunar cells show abundant, clear cytoplasm, a polylobated nucleus showing fine chromatin and much less conspicuous nucleoli (Figure 1.1). They may be found singly or in small aggregates, sometimes forming large, syncytial sheets in which there are areas of necrosis.

The diagnosis of NS also requires the identification of nodule formation and some evidence of banding sclerosis and intranodal bands of sclerosis (Lukes et al 1971). The number and morphology of the classical RS cells and lacunar cells can vary widely as can the cellular back-ground - ranging from lymphocyte predominance to lymphocyte depleted with varying numbers of plasma cells, histiocytes and eosinophils. The extent of sclerosis present is also very variable.

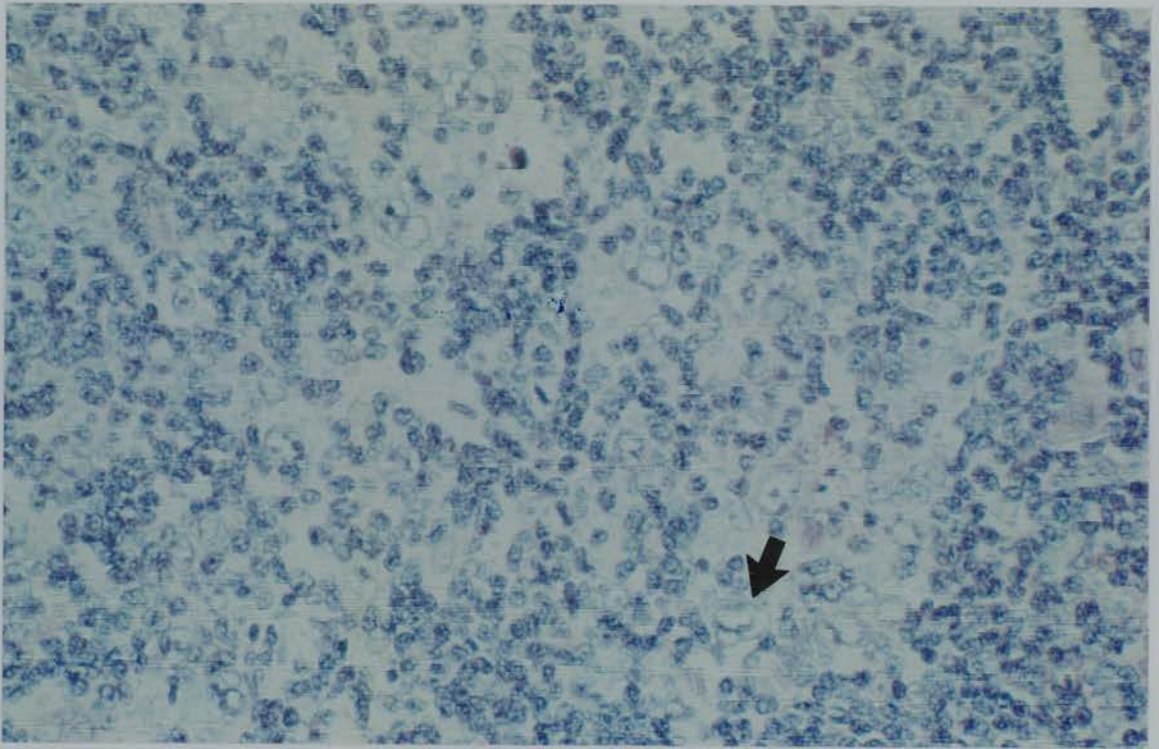
## **KEY FOR FIGURE 1.1**

Arrow indicates an L and H cell.

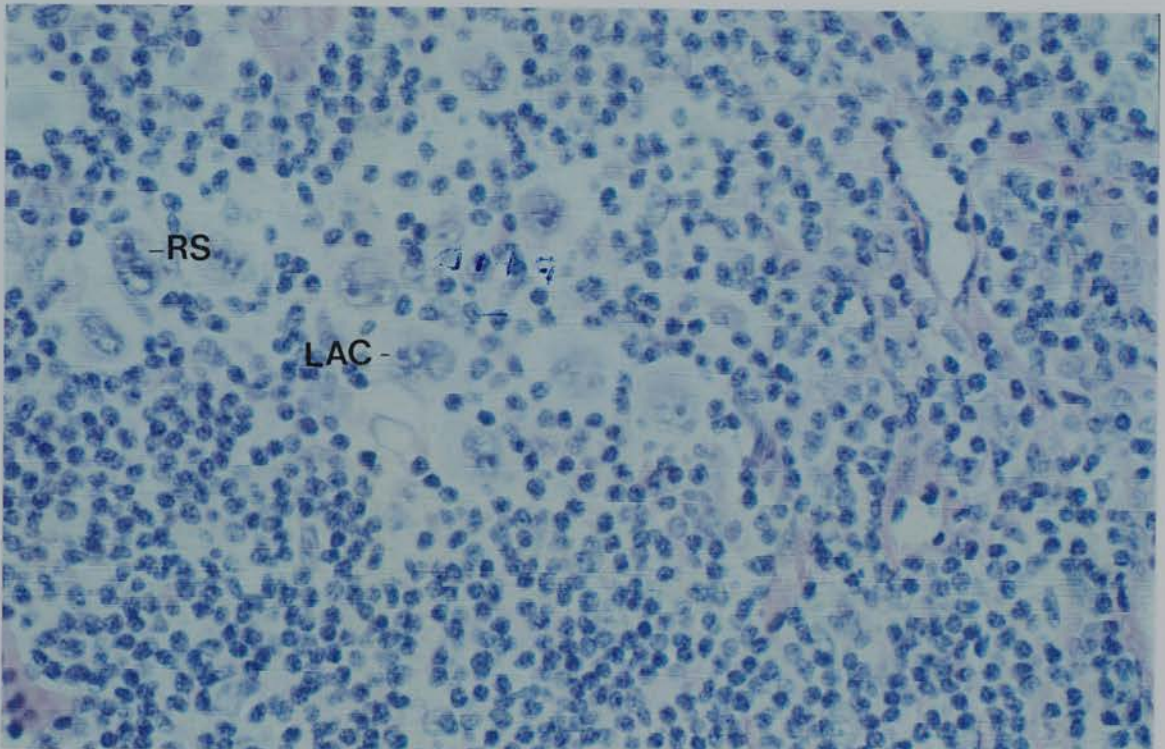
RS - classical Reed-Sternberg cell

Lac - Lacunar cell





Lymphocyte Predominant Hodgkin's disease showing L & H cells. Mag x250



Nodular Sclerosing Hodgkin's disease showing classical RS cells and lacunar cells. Mag x250

**Figure 1.1** The Histological Appearance of Lymphocyte Predominant and Nodular Sclerosing Hodgkin's Disease

NSHD can be sub-divided into 2 prognostic groups depending on histological features. In NS1, the nodules show a predominance of lymphocytes, and the prognosis is better than any other sub-type apart from LPHD. In NS2, more than half the nodules show lymphocyte depletion and marked pleomorphism of the RS cells with prognosis worse than MCHD and only marginally better than LDHD. Type 2 NSHD makes up about 30% of the NS cases and is the predominant sub-type over the age of 50 (Bennett et al 1985).

### Mixed Cellularity Hodgkin's Disease

This is the second most common sub-type, making up between 20 - 40% of cases. It mostly occurs in young adults but it is the most common sub-type encountered in later life. This sub-type is more commonly found in males and usually presents in the neck nodes (Colby et al 1981, Mauch et al 1993).

There can be difficulties in defining the MC sub-type from the other sub-types as it contains numerous classical RS cells but both Lacunar cells and the Pleomorphic variant of the RS cell may also be present. The Pleomorphic variant shows even more bizarre nuclear multilobation, some also show gigantic nucleoli. The composition of the cellular infiltrate present in the MC sub-type is very varied from areas of lymphocyte depletion to areas of lymphocyte predominance, but usually also includes eosinophils, neutrophils, plasma cells and histiocytes (Figure 1.2). The MC sub-type may also show disorganised sclerosis unlike the banded , organised sclerosis seen in the NS sub-type.

### Lymphocyte Depleted Hodgkin's Disease

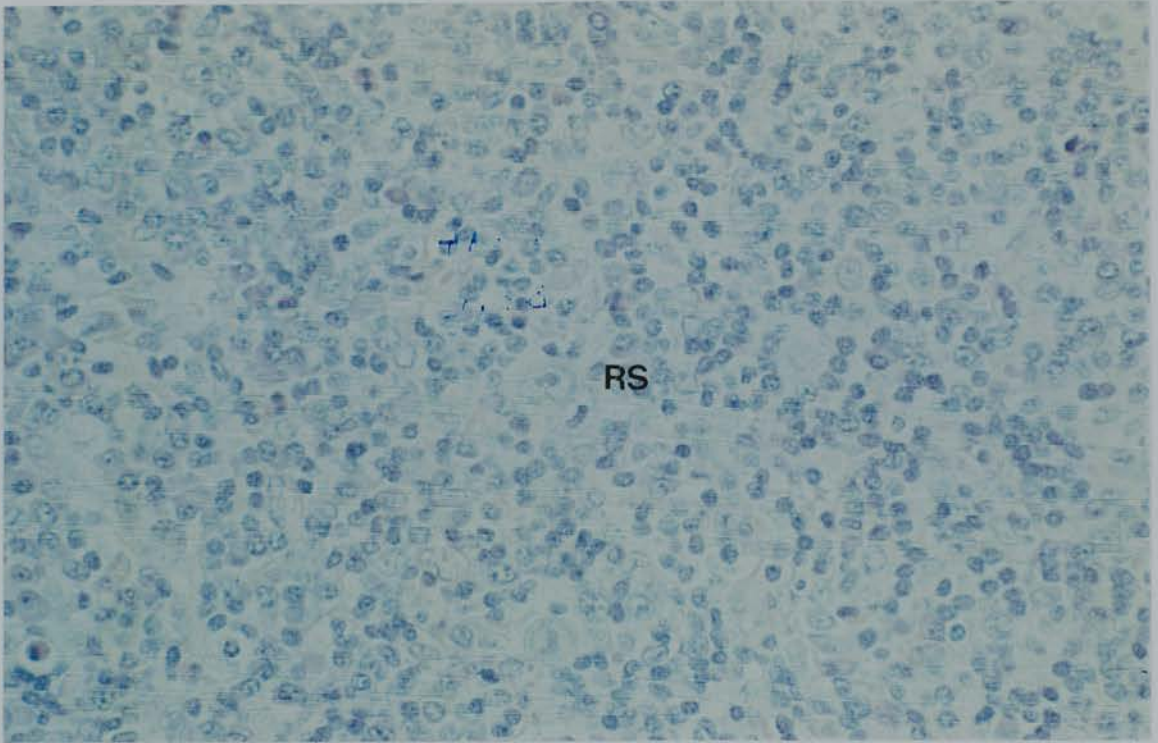
This is the least common sub-type accounting for about 4% of cases (Bennett et al 1985). It is mainly a disease of late adult life. There are 2 sub-types of LDHD - Reticular and Diffuse Sclerosis (Lukes & Butler 1966, Lukes et al 1966). In the Reticular form, there is a stromal response similar to that found in the MC sub-type, but there is a predominance of the Pleomorphic RS cell variant. In the Diffuse Sclerosis sub-type, Pleomorphic and Classical RS cells are set in a hypo-cellular back-ground of disorganised sclerosis (Figure 1.2).

The Rye classification of Hodgkin's disease has remained stable for 25 years and is still considered to be clinically and pathologically relevant (Bennett et al 1991). However, the accurate distinction of the 4 histological sub-types can be difficult. Also, it is necessary to be aware that there is considerable morphological over-lap between certain types of Non-Hodgkin Lymphomas and Hodgkin's disease, i.e. MCHD and the peripheral T Cell Lymphomas and LDHD and the Ki-1 positive Large Cell Anaplastic Lymphomas, to avoid misdiagnosis as Hodgkin's disease (Agnarsson & Kadin 1988, Harris 1992).

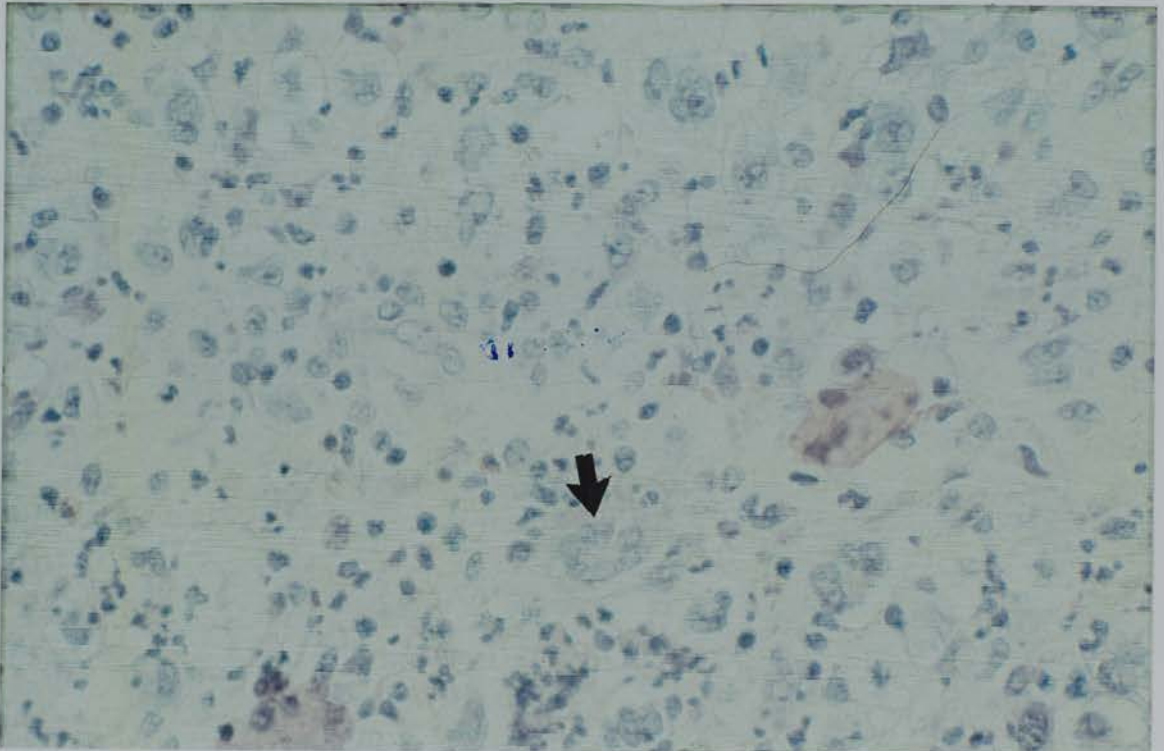
Another problem with the Rye classification is the concept that the 4 sub-types are inter-related with possible progression from good to poor prognosis sub-types i.e. transition from LP to MC to LD. It is now clear that LPHD is a biologically distinct entity that does not progress to other sub-types but may rarely transform to a high grade B cell lymphoma (Poppema 1992).

Recently, there has been a proposal for a new classification system for lymphoid neoplasms called the Revised European American Lymphoma





Mixed Cellularity Hodgkin's disease with a lymphocyte predominant cellular background showing a classical RS cell. Mag x250



Lymphocyte Depleted Hodgkin's disease showing a hypocellular background almost devoid of lymphocytes. A pleomorphic RS cell is seen. Mag x250

**Figure 1.2    Histological Appearance of Mixed Cellularity and Lymphocyte Depleted Hodgkin's Disease**

## **KEY FOR FIGURE 1.2**

RS - classical Reed-Sternberg cell

Arrow indicates a pleomorphic Reed-Sternberg cell



(REAL) classification (Chan et al 1994). Under this classification system, an extra HD sub-type has been entered called Lymphocyte-Rich Classical Hodgkin's Disease. This sub-type shares the clinical features of NS or MCHD and the immunophenotype of NSHD. Histologically, diffuse effacement of architecture is seen, with infrequent classical RS cells (rather than L and H cells) scattered in a lymphocyte-rich back-ground (Chan et al 1994). However this is a provisional entity which may be deleted or become established as more information is collected.

#### **1.1.4 Histogenesis of the Reed-Sternberg Cell**

Although much progress has been made in the treatment of Hodgkin's disease, and it has been the subject of many studies, the histogenesis of the RS cell remains unclear.

Immunohistochemical staining of HD in general shows that RS cells commonly express the CD30 activation marker (Schwab et al 1982), the granulocyte-associated antigen CD15 (Hsu & Jaffe 1984), MHC Class II, CD71 (the transferrin receptor) and the Tac antigen (IL-2 receptor - CD25) demonstrating the activated state of the RS cells (Hugh & Poppema 1992), and adhesion molecules such as CD54 (ICAM-1), CD58 (LFA-3) and CD44 (Paeitta 1992). They are usually negative or stain very weakly with the leucocyte common antigen (LCA - CD45) and the epithelial membrane antigen (EMA). The exception to this rule is the L and H variant of the RS cell in the Lymphocyte Predominant sub-type which stains additionally for CD45 and EMA. Variable results have been reported with antibodies against CD30 (Ki-1, Ber-H2). Although most cases show CD30 (Ki-1)

staining of L & H cells in frozen sections, Ber-H2 stains less than 50% of cases in paraffin sections. CD15 staining of the L and H cells however is unusual in NLPHD (Nicholas et al 1990).

Cell kinetic studies in HD are comparatively limited (Peckham & Cooper 1969). However, with the use of specific markers for RS/H cells and the monoclonal antibody Ki-67 which stains a cell cycle associated antigen expressed in all phases except G<sub>0</sub>, both Gerdes et al (1987) and Falini et al (1987) demonstrated a high percentage of RS/H cell proliferation (53-98% Ki-67 positive RS/H cells). More recently, studies using anti-PCNA/cyclin (a cell cycle regulated protein with increased expression in G1 and S phase, correlating to DNA synthesis) (Otago et al 1987) antibodies have also reported the high percentage of apparently cycling RS/H cells (Benjamin & Gown 1991, Schmid et al 1992).

Conflicting evidence over the years has suggested a monocyte/macrophage (Kadin et al 1978), dendritic reticulum cell (Curran & Jones 1977) and interdigitating cell origin for the RS cell (Hsu et al 1985).

Kadin et al (1978) supported the view that the RS cell is derived from the macrophage. They argue against a B cell origin as they found that cytoplasmic immunoglobulin of individual RS cells was always polyclonal (expresses both kappa and lambda light chains) making intracellular synthesis of the immunoglobulin by the RS cells unlikely. Using *in vitro* studies, they concluded that exogenous immunoglobulin is internalised (probably via an Fc receptor) by the RS cells and favoured a macrophage origin for the cell.

However, Hsu et al (1985) and Watanabe et al (1982) found that RS cells failed to react with macrophage markers such as  $\alpha$ 1-antitrypsin or lysozyme. Meiss et al (1986) found occasional positivity with  $\alpha$ 1-antitrypsin

but this was also found in some B and T cell lymphomas. RS cells also failed to react with monocyte markers such as OKM1, MO-2, Leu-3 etc (Hsu et al 1985).

Curran & Jones (1977) suggested an origin from follicular dendritic cells with the presence of immunoglobulin resulting from absorption. However, Hsu et al (1985) found that the RS cells also failed to react with the follicular dendritic cell markers such as DRC-1. Hsu et al (1985) also found negativity with a wide range of T and B cell markers and suggested an origin for RS cells from interdigitating reticulum cells from a similar antigenic phenotype, and the frequent involvement of the T cell zones in Hodgkin's disease (where the interdigitating reticulum cells are localised). However, Meiss et al (1986) and Watanabe et al (1982) both found negativity with the interdigitating reticulum cell marker - S100 protein.

The current consensus is that the RS cell is an activated lymphoid cell with an aberrant phenotype (although proposals of a follicular dendritic cell origin are currently gaining popularity). Distinct similarities between RS cells and transformed lymphocytes (large with vesicular nuclei and prominent nucleoli) have been demonstrated ultrastructurally (Biniaminov & Ramot 1974). The finding that lymphokines including IL-4, IL-5, IL-6 and IL-9 are produced by HD tissues also supports the argument that RS/H cells derive from lymphocytes.

There is however no consensus as to whether the RS cell is of T or B cell origin.

### Nodular LPHD as a B Cell Lymphoma

The exception to this is the Nodular Lymphocyte Predominance sub-type which is now widely accepted to be a B cell lymphoma. Investigators favouring a T cell origin for the RS cell treat the LP sub-type as the exception and either omit it from their studies or demonstrate its negative staining with any T cell marker. It can be distinguished immunohistologically from the other sub-types (LCA positive, CD15 negative, back-ground population of polyclonal B cells) and Timens et al (1986) proposed that it was actually a germinal centre lymphoma - the result of abnormal proliferation of cell types present in the normal follicles and germinal centres, the L and H cells being transformed B cells. Another feature which distinguishes LPHD is the much higher CD57 reactivity of the back-ground T cells, with characteristic 'ringing' of the L and H cells (Kamel et al 1993). The L and H cells have been shown to contain cytoplasmic J chain, a polypeptide synthesised exclusively by B cells (Stein et al 1986) and these CD57 positive background T cells may be relevant as functional studies have shown that CD57 positive T cells may play a role in the regulation of Ig production by B cells (Banerjee et al 1988). However, the biological significance of these T cells in NLPHD remains uncertain.

In several studies, the L and H cells have been shown to stain with B cell markers such as L26 (CD20) and MB2 in over 90% of cases (Norton & Isaacson 1987, Hall et al 1988, Nicholas et al 1990, Poppema 1992a). It should be noted that in most cases, the L and H cells show polytypic (presumably endocytosed) immunoglobulin (Ig) light chain expression. However, it has been reported that in some cases L and H cells express monotypic kappa or lambda light chain. This provides strong evidence for the monoclonality and therefore neoplastic nature of these L and H cells and

their B cell origin (Schmid et al 1991a, Momose et al 1992). This was confirmed by *in situ* hybridisation for light chain mRNA (Hell et al 1993). However, most studies have failed to demonstrate Ig gene rearrangements (Algara et al 1991, Said et al 1991).

The cellular origin of the RS cell is not so clear cut in the other subtypes of Hodgkin's disease (NS,MC,LD) with conflicting phenotypic and genotypic studies.

#### Phenotypic Studies in Other HD Sub-types (NS, MC, LD)

The fact that initial lesions often arise in the T cell zone of the affected lymph node or spleen, has prompted some investigators to suggest that the RS cell may have its origin in a transformed T lymphocyte, perhaps antigenically altered by a viral infection. They hypothesise that HD is an interaction between the transformed T lymphocyte and the normal T cells and reactive B cells (Biniaminov & Ramot 1974, Berard et al 1980). The discovery by Samoszuk & Nansen (1990) that RS cells strongly express IL-5 mRNA prompted them to suggest that RS cells may be functionally related to T cells. Some investigators have found evidence of RS cells expressing T cell antigens (including CD3 which is highly specific for T cells). Kadin et al (1988) found that in 8/30 cases of Hodgkin's (NS,MC), the RS cells stained positively with T cell markers (CD2,3,4). Many investigators argue that it is difficult to distinguish RS cell staining with T cell markers from the small T lymphocytes surrounding them, but Kadin et al confirmed the surface antigen staining of RS cells by the deposit of a dark linear reactive product along the cell membrane in electron micrographs. They suggested a T cell origin for RS/H cells in some cases of HD.



Dallenbach and Stein (1989) found positive staining of a sub-population of the RS/H cells in 14/65 cases of Hodgkin's (NS,MC,LD) with a T cell receptor  $\beta$  chain antibody. The staining was usually localised on the membrane, presumably reflecting endogenous TcR  $\beta$  chains, although it was stated that positive staining of the RS cells could not be determined precisely in some cases because of the surrounding TcR  $\beta$  positive T cells. The TcR  $\beta$  positive staining was backed up by positive staining with T cell (CD2,3,4) but not B cell (except LPHD) markers. Falini et al (1987) performed immunostaining on lymph node cytopins to distinguish the RS cell staining, although it was stated that the RS cells were still frequently surrounded by a rim of T lymphocytes. They found positive cytoplasmic and/or membrane staining of RS cells with T cell markers (CD3,4,5,6,8) in 8/30 cases (MC,LP,LD sub-types).

It is clear that these reports are not pointing to an exclusive T cell origin for the RS cell. Those who included B cell markers on their panel found the presence of T and B cell markers in the cases of Hodgkin's Disease studied (Casey et al 1988, Oka et al 1988, Agnarrson & Kadin 1989). In fact most of these investigators favouring a T cell origin for RS cells conclude that Hodgkin's Disease is a phenotypically heterogeneous disease of either T (most common) or B (less common) cell origin.

Many studies have shown positive staining of RS/H cells with immunoglobulin of both kappa and lambda light chain sub-types (Curran & Jones 1978, Poppema et al 1978, Isaacson 1979, Stein et al 1981, Lauritzen et al 1992, Hell et al 1993) and therefore polyclonal, making intracellular synthesis by the RS cells unlikely. Some argue that the uptake of exogenous polyclonal immunoglobulin by the RS cells masks detection of endogenous immunoglobulin of a monotypic nature (Isaacson 1979).

However, one study found that these RS cells do not stain for J chain providing strong evidence that the immunoglobulin was not synthesised endogenously by the RS cell and therefore not of B cell origin (Isaacson 1979).

*In situ* hybridisation studies have also failed to demonstrate immunoglobulin light chain mRNA in RS cells (NS,MC sub-types) (Ruprai et al 1991) even when a more sensitive method using digoxigenin labelled probes able to detect low to medium copy numbers of mRNA species was employed (exception was 1 case of MCHD) (Hell et al 1993).

However, many studies have shown immunostaining with B cell markers in RS cells of all sub-types of Hodgkin's. Agnarsson & Kadin (1989) found in NSHD that 100% of cases expressed CD20, and 33% of cases expressed CD22. Norton and Isaacson (1987), investigating L26 reactivity of RS cells, found positive staining in 50% of MCHD cases and in 20% of NSHD cases. In a study by Hall et al (1988), 43 cases of Hodgkin's were studied, no case showing immunoreactivity with any T cell markers - however, a proportion of each sub-type (MC, NS, LD) was positive with the B cell markers L26 and MB2. Schmid et al (1991b) found in 87% of cases (MC, NS sub-types) that RS cells were reactive with at least one B cell marker (CD19, CD20, CD22, CD75, MB2). O'Grady et al (1994a) demonstrated strong expression of the CD40 B cell activation antigen in 23/33 cases of Hodgkin's (MC, NS, LD sub-types) studied - of the 23 CD40 positive cases, 10 co-expressed one or more B cell markers.

Imam et al (1990), used an anti-BLA.36 antibody which recognises a 36kD glycoprotein antigen termed B lymphocyte antigen (BLA.36). This antigen is expressed on normal and malignant B cells but not on T cells or macrophages. Reed-Sternberg cells of all sub-types of HD were found to

express this antigen strongly, lending support to the B cell origin of RS/H cells.

A recent report identified a novel B cell activation antigen recognised by monoclonal antibody FUN-1 (Nozawa et al 1993). FUN-1 recognises a 75kD protein present on activated B cells, EBV-transformed B cells, large B cell lymphomas and LCAL. Resting lymphocytes, activated T cells and low grade B cell leukaemias do not react with the antibody. Reed-Sternberg cells of all sub-types of HD were found to express this antigen, also lending support to the B cell origin of RS/H cells.

Therefore, a proportion of the HD sub-types other than LPHD are found to stain with B cell markers.

Some investigators simply take the view that RS cells represent malignant counterparts of lymphoid cells of either T or B cell origin that are in an activated state (Stein et al 1985).

Another approach used to define the cellular origin of Hodgkin's disease is genotypic analysis of immunoglobulin or T cell receptor gene rearrangements.

#### Genotypic Studies in Other HD Sub-types (NS, MC, LD)

During the differentiation of B and T cells, the immunoglobulin (Ig) and T cell receptor (TcR) genes undergo specific recombinations. One out of several V (variable), D(iversity) and J(joining) genes are recombined together to form an active V-D-J complex for the immunoglobulin heavy and T cell receptor beta chains, and a VJ complex for immunoglobulin light and T cell receptor alpha chains. The T cell receptor gamma and delta genes also recombine in this way and are expressed on a small fraction



(approximately 10%) of T cells. The rearrangements of the Ig and TcR genes are individual for each T and B cell clone. Therefore, these rearrangements can be used as markers for the clonal origin of a sub-set of T or B cells in a heterogeneous population, and since tumours are characterised as a clonal proliferation of cells which display the same receptor molecules, these rearrangements are good tumour-specific markers. The Ig and TcR gene rearrangements are easily identified by Southern blotting.

Several groups have investigated this, and again conflicting ideas have arisen.

In gene rearrangement studies in Hodgkin's Disease, different investigators find Ig only, TcR only, both or none. Greisser et al (1987) found TcR beta rearrangements in 4/22 cases and TcR gamma rearrangements in 15/22 cases, including 3 Lymphocyte Predominant cases. They also found IgH rearrangements in 2/22 cases, both Lymphocyte Depleted, and conclude that different rearrangements patterns may be associated with different sub-types. However, in the majority of studies, TcR rearrangements have not been detected in Hodgkin's disease (Knowles et al 1986, Weiss et al 1986, Sundeem et al 1987, Roth et al 1988, Gledhill et al 1990, Tesch et al 1990).

Roth et al (1988) and Knowles et al (1986) found no rearrangements of Ig or TcR genes in their studies concluding that RS cells do not represent clonal B or T cell populations.

The majority of studies detect some Ig gene rearrangements in Hodgkin's Disease. Tesch et al (1990) found rearrangements of JH genes in 2/7 cases and of the Jk gene in another case. No TcR rearrangements were found. Gledhill et al (1990) found both IgH and IgL gene

rearrangements in 3/9 cases, all 9 cases had germline TcR beta and gamma genes. One could argue that the lack of detectable TcR gene rearrangements in many studies could be due to insufficient numbers of RS cells in the biopsies, but Sundeem et al (1987) and Weiss et al (1986) both used cases with a high number of RS cells, either selected solely on the basis of high RS cell content, or enriched for RS cells by cell separation techniques. No TcR gene rearrangements were detected in either of these studies. However, Sundeem et al (1987) found at least one Ig gene rearrangement in 3/5 cases and Weiss et al (1986) in 6/7 cases.

These results suggest that clonal cell populations with uniform Ig gene rearrangements are present in the tissue in some cases of Hodgkin's Disease. The fact that these rearrangements are present in enriched RS cell suspensions, and increased in cases with high RS cell number, suggests that the rearrangements are taking place in the RS cells.

Therefore, both phenotypic and genotypic studies have given conflicting results on the histogenesis of the RS/H cells. Hence, a new mode of investigation is necessary.

## **1.2 The Non-Hodgkin's Lymphomas**

### **1.2.1 Clinical Features**

The malignant lymphomas other than Hodgkin's lymphoma are commonly referred to as the non-Hodgkin's lymphomas (NHL). They are a heterogeneous group of lymphoproliferative malignancies whose clinical behaviour and prognosis vary widely according to histologic sub-type, stage and bulk of disease. The most common mode of presentation is lymphadenopathy, although they can arise extra-nodally i.e. skin, GI tract etc. Other presenting features are haematological cytopenia, night sweats, fever, weight loss, hepatosplenomegaly, abdominal masses or compression of internal organs. The NHL are the 8th most commonly diagnosed malignancy. In the Western world, a large majority of NHL appear to arise from B lymphocytes or their precursors. Practically all the malignant lymphomas occurring in child-hood (except for HD) are high grade blast cell neoplasms. All the common low grade B cell NHL are neoplasms of later life, only rarely encountered under the age of 25 and practically unknown under the age of 18 (O'Reilly & Connors 1992).

The treatment chosen for NHL is determined by the age and health of the patient, the stage of the lymphoma which is determined by the Ann Arbor classification system and the particular histologic sub-type. Surgery is curative in rare patients with localised extra-nodal NHL but is rarely recommended as the sole treatment. Radiotherapy alone is usually used with Stage I disease, combination chemotherapy with or without radiotherapy is given for the more advanced stages of the disease (Armitage et al 1993).

### **1.2.2      Classification**

Unlike Hodgkin's disease, NHL can be classified into T or B cell lymphomas, low or high grade. Over the years, numerous classification systems have been employed - one of the earliest being the Rappaport Classification (Rappaport 1966). The 2 most commonly used classifications today are the Working Formulation (Nat Can Inst Study 1982) and the modified Kiel Classification (Lennert 1978, Stansfeld et al 1988) - the latter being the preferred terminology in most cases (Table 1.1).

This study uses the up-dated Kiel classification system, however the recently established REAL (Revised European American Lymphoma) classification may be the system used in the future. The REAL system includes extra-nodal lymphomas, specifically excluded from the Kiel classification. It produces an international consensus on terminology, commonly used terms are retained with minimal creation of new terminology. The classification is a list of biological entities as defined by multi-parameter studies (Chan et al 1994).

### **1.2.3      T Cell NHL**

T cell NHL are a heterogeneous group of tumours that are difficult to identify and classify (Wright 1986). They are relatively uncommon and only account for 10 - 15% of the NHL (Weiss et al 1985, Krajewski et al 1988). The neoplastic cells express one or more of the pan T cell antigens (although in some cases, particularly in large cell categories, loss of pan T cell antigens

**Table 1.1 Up-dated Kiel Classification of Non-Hodgkin's Lymphomas**

<b>B</b>	<b>T</b>
<p><b><u>Low Grade</u></b></p> <p>Lymphocytic - chronic lymphocytic and prolymphocytic leukaemia; hairy cell leukaemia</p> <p>Lymphoplasmacytic/cytoid (LP immunocytoma)</p> <p>Plasmacytic</p> <p>Centroblastic/centrocytic</p> <ul style="list-style-type: none"> <li>- follicular <math>\pm</math> diffuse</li> <li>- diffuse</li> </ul> <p>Centrocytic</p>	<p><b><u>Low Grade</u></b></p> <p>Lymphocytic - chronic lymphocytic and prolymphocytic leukaemia</p> <p>Small, cerebriform cell - mycosis fungoides, Sezary's syndrome</p> <p>Lymphoepitheloid (Lennert's lymphoma)</p> <p>Angioimmunoblastic (AILD, LgX) T zone</p> <p>Pleomorphic, small cell (HTLV-1 <math>\pm</math>)</p>
<p><b><u>High Grade</u></b></p> <p>Centroblastic</p> <p>Immunoblastic</p> <p>Large Cell Anaplastic (Ki-1+)</p> <p>Burkitt's Lymphoma</p> <p>Lymphoblastic</p>	<p><b><u>High Grade</u></b></p> <p>Pleomorphic, medium and large cell (HTLV-1 <math>\pm</math>)</p> <p>Immunoblastic (HTLV-1 <math>\pm</math>)</p> <p>Large Cell Anaplastic (Ki-1+)</p> <p>Lymphoblastic</p>
<p>Rare Types</p>	<p>Rare Types</p>



can be found) as well as either CD4 or CD8 - the majority being CD4 positive (Krajewski et al 1988, Jaffe et al 1992).

The lymphomas of T cell lineage can be divided into 2 main groups based on their origin from immature or mature T cells. The lymphoid neoplasm of immature T cells is the lymphoblastic NHL whereas all the lymphomas derived from mature T cells can be grouped as 'peripheral T cell lymphomas' (Waldron et al 1977). However, mycosis fungoides and Sezary's syndrome correspond to mature T cells which home in and reside in the skin and as such represent a special sub-set of peripheral T cell lymphomas (Grogan et al 1985, Horning et al 1986).

#### **1.2.4      B Cell NHL**

The B cell NHL constitute the majority of the NHL. The cells of B-NHL are lymphocytes or lymphoblasts with different morphologies that characteristically express membrane Ig. Some may secrete Ig, but for the most part do not, and Ig secretion when seen is usually limited. B-NHL cells are related to B cells that have completed V-(D)-J Ig gene rearrangements in the bone marrow but which have not matured into plasma cells (Potter 1992).

NHL represent neoplastic counterparts of normal reactions taking place in the lymphoid tissues, each malignant cell type has its benign analogue in the non-neoplastic lymph node - this concept has been worked out in some detail for the B-NHL. The majority of NHL are derived from follicle centre cells.

2 main cell types are found in the follicle centre - termed centrocytes and centroblasts. Centrocytes vary in size from a little larger than lymphocytes to double that size and may have indented or cleaved nuclei with dark chromatin and no nucleoli. Centroblasts have round, pale-staining nuclei which are not indented and which usually contain several prominent nucleoli apposed to the nuclear membrane. The distribution of centroblasts and centrocytes is uneven within the follicles dividing it into so-called light and dark zones. Centroblasts are concentrated in the basal dark zone and centrocytes in the apical light zone. Most experimental evidence supports the assumption that in the development of a germinal centre, the centroblasts appear first and the centrocytes develop from them at a later time (Nieuwenhuis & Keuning 1974). Indeed the centroblasts appear to be the dominant cell type present in early germinal centre formation. There is no doubt that these 2 cell types are 2 different stages in the B lymphocyte life cycle and the fact that one transforms into another is supported by the presence of intermediate cell types within the fully established germinal centre. Other lymphoid cells in the follicle centre include immunoblasts, occasional plasma cells and small lymphocytes. Immunoblasts have a large, 'vesicular' nucleus with a prominent, usually central, nucleolus. The resemblance of such cells to plasma cells increases as the cell matures.

In addition to follicle centre cells, other B cell subsets may be involved in lymphoma such as the CD5 positive mantle zone cell and the CD5 negative marginal zone cell.

The morphological relationship of the lymphoid cells in the follicle centre and their related lymphomas are shown in Figure 1.3. This is based on morphological, immunological and experimental studies (Stein et al 1980, Van der Valk & Meijer 1988). Lymphomas associated with the plasma cell

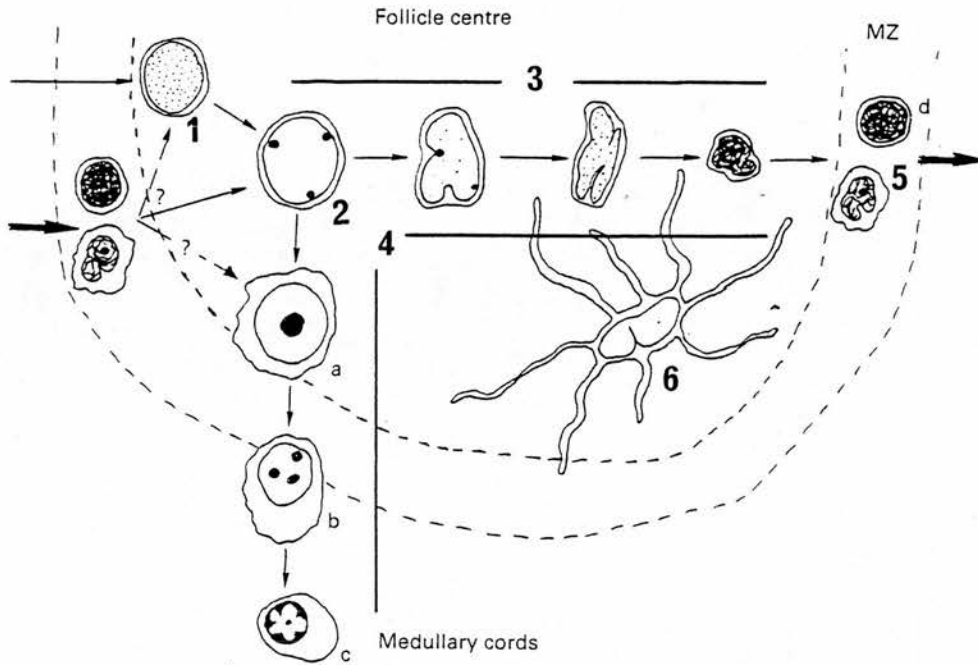
reaction are shown in Figure 1.4. This is based on morphological and immunological studies (Weissman et al 1978, Harris & Bahn 1985, Van der Valk & Meijer 1988).

The various sub-types of NHL (all B-NHL) encountered in this project will be described in some detail in the following sections.

### **1.2.5      Malignant Lymphoma: Centroblastic-Centrocytic Follicular (ML Cb-Cc Foll)**

This low grade B-NHL is the commonest NHL accounting for 25-50% of cases in Europe and North America (Lennert 1981). ML Cb-Cc almost always shows a follicular pattern in the affected lymph node, although a diffuse pattern is also seen (ML Cb-Cc Diffuse). The neoplastic follicles are composed of a variable mixture of centrocytes and centroblasts usually with the former predominating, together with the non-neoplastic cells that are found in the normal germinal centre - dendritic cells, macrophages and T cells (Stansfeld 1992a) (Figure 1.5).

As with all the other low grade B-NHL, ML Cb-Cc Foll is a neoplasm of adults, the incidence in the 2 sexes is almost equal. The usual mode of presentation is lymphadenopathy, frequently found to be wide-spread at the time of presentation (Jones et al 1973). The neoplasm occupies most of the lymph node and may extend through the capsule into the surrounding fat. There is always a certain number of centroblasts in the neoplastic follicles, although they are sometimes very scanty (1-2%), but usually make up 3-10%. Generally, these centroblasts resemble the centroblasts in the normal germinal centre (Levine & Dorfman 1975), but occasionally there are some

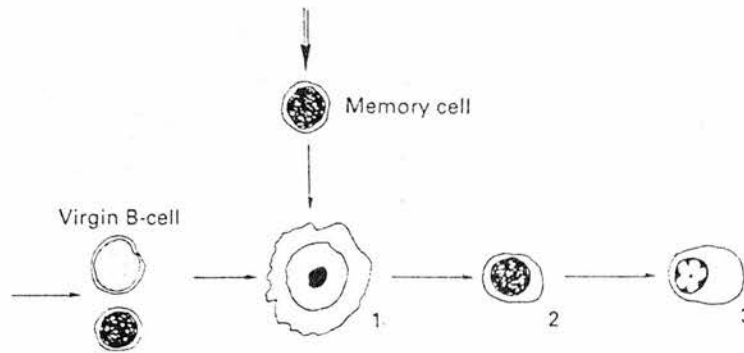


**Figure 1.3 Follicle Centre Cells and Corresponding Lymphomas**

The thick arrows indicate recirculation. As shown on the left, the route a recirculating (memory) cell will take after renewed contact with antigen is uncertain. **MZ** = mantle zone; **a** = immunoblast; **b** = plasmablast; **c** = plasma cell; **d** = recirculating lymphocyte.

The lymphomas arising from the depicted cells are : **(1)** lymphoblastic; **(2)** centroblastic; **(3)** centroblastic/centrocytic; **(4)** polymorphic immunocytoma; **(5)** mantle zone lymphoma (REAL classification, centrocytic in Kiel); **(6)** dendritic reticulum cell sarcoma

Adapted from Van der Valk & Meijer 1988



**Figure 1.4 Plasma Cell Reaction and Corresponding Lymphomas**

The thick arrow indicates recirculation.

The lymphomas arising from these cells are: **(1)** B-immunoblastic; **(2)** lymphoplasmacytoid/-cytic; **(3)** plasmacytoma / multiple myeloma

Adapted from Van der Valk & Meijer 1988.



blast cells with very prominent central nucleoli more like those of immunoblasts. Very occasionally there may be a highly atypical binucleate form resembling RS cells. The centrocyte is the predominant cell type and within a single case there is often variation in the size - sometimes medium-sized or large centrocytes predominate instead of the usual small centrocyte.

Surface immunoglobulin of a monoclonal type is a regular feature of the neoplastic centrocytes - occasionally there may be evidence of cytoplasmic immunoglobulin in such tumours as well (Stansfeld 1992a).

ML Cb-Cc Foll are consistently associated with the t(14;18) translocation which results in inappropriate expression of the bcl-2 protein (Cotter 1990). However, the bcl-2 protein can be expressed in follicular lymphoma (90% of cases) in the absence of the 14;18 translocation (Pezzella et al 1990, Pezzella et al 1991).

There is often a surprisingly large number of reactive T cells present in ML Cb-Cc Foll. Most take the view that this is merely reactive and associated with indolent behaviour of the B cell neoplasia and good prognosis (Jaffe et al 1984).

ML Cb-Cc Foll lymphomas that contain a larger number of centroblasts have a worse prognosis than those composed mainly of centrocytes (Warnke et al 1977). Transformation from a low grade tumour into a high grade centroblastic lymphoma is seen in about 40% of cases of ML Cb-Cc Foll (Lennert 1981). A loss of follicular pattern and a marked increase in the number of centroblasts especially if they are found in large, solid clumps or if many of the blast cells are atypical signals the transformation into a high grade centroblastic lymphoma. Very occasionally, transformation into a B immunoblastic lymphoma is seen (Stansfeld 1992a).

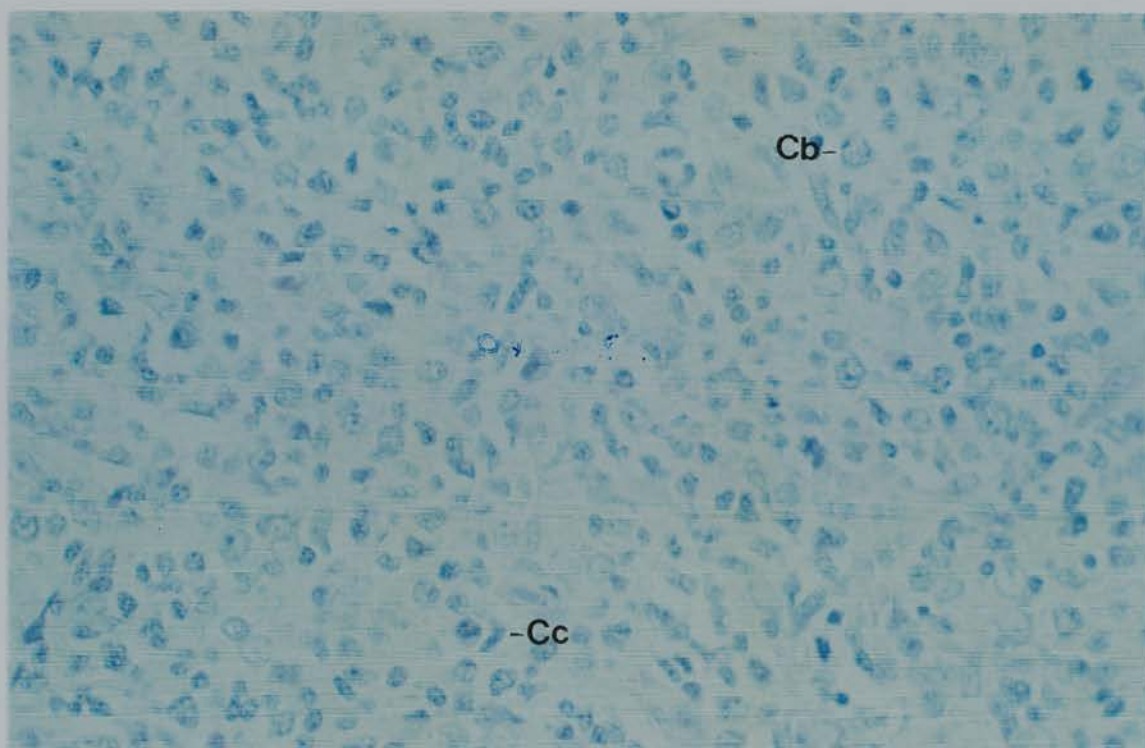
### 1.2.6 ML Centroblastic (ML-Cb)

This tumour accounts for around 40% of NHL. It is the most common type of high grade B cell lymphoma in the Western world and is slightly more common in males (Hui et al 1988). It is usually categorised as an intermediate grade in the Working formulation and high grade in the Kiel classification - it is not as aggressive as the high grade immunoblastic and lymphoblastic lymphomas but certainly more aggressive than the low grade B cell lymphomas.

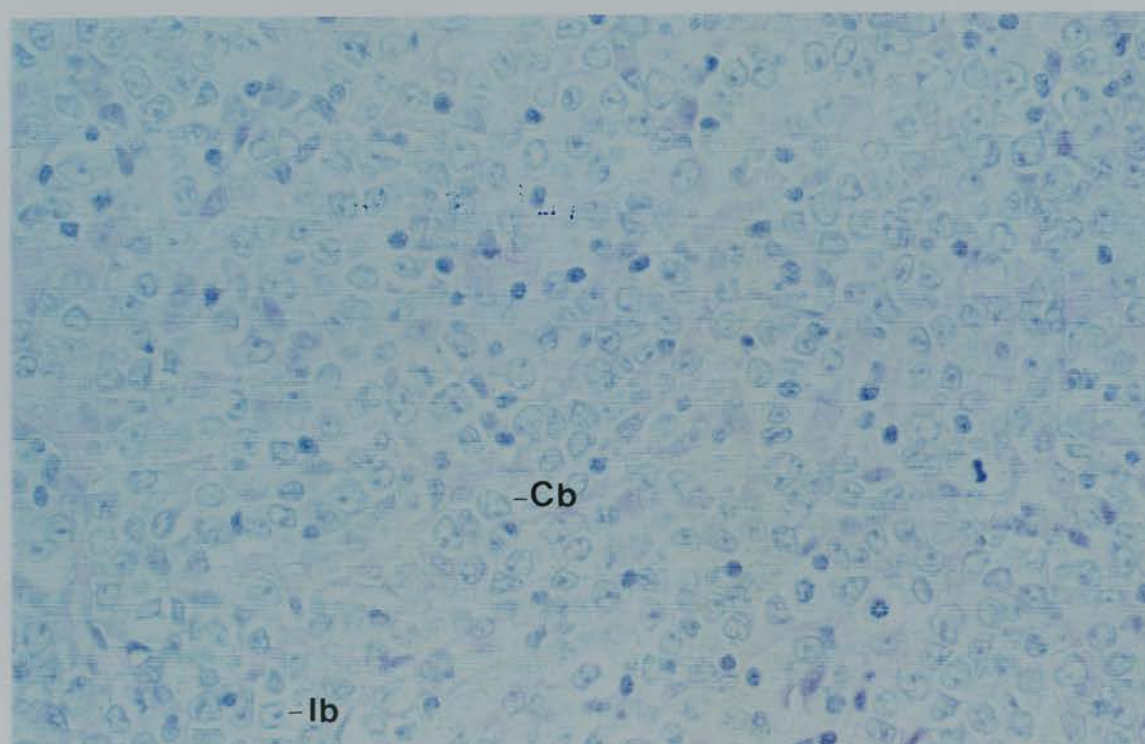
The centroblastic lymphoma can arise *de novo* as a high grade lymphoma or from the transformation of a pre-existing low grade ML Cb-Cc lymphoma as stated above. Centroblastic lymphomas may occasionally exhibit a follicular pattern, but are more usually follicular and diffuse, or diffuse. They are usually composed of a mixture of centroblasts and immunoblasts with scanty centrocytes (called the polymorphic sub-type) but occasionally almost pure centroblastic tumours are seen (the monomorphic sub-type). Also found is the multi-lobated sub-type in which a large proportion of the blast cells have bizarre, multi-lobated nuclei and the centrocytoid sub-type in which the majority of the neoplastic cells resemble large centrocytes, although some typical centroblasts are also present (Stansfeld 1992b).

Many of the tumours show monotypic surface immunoglobulin - occasionally cytoplasmic immunoglobulin is also found (Stansfeld 1992b).

There is no sharp border between the monomorphic and polymorphic variants, or even between centroblastic and immunoblastic lymphoma - the main problem is distinguishing between a polymorphic centroblastic lymphoma and a B immunoblastic lymphoma (Hui et al 1988) (Figure 1.5).



Cb-Cc follicular lymphoma (Case J) showing both centrocytes and centroblasts. Mag x250



Centroblastic lymphoma (Case G) comprised mainly of centroblasts, with a few immunoblasts.

**Figure 1.5** Histological Appearance of Follicular and Centroblastic Lymphoma

## **KEY FOR FIGURES 1.5 AND 1.6**

Cb - centroblast

Cc - centrocyte

Ib - immunoblast



### **1.2.7      ML Immunoblastic - B Type (ML Ib-B)**

These are large cell, high grade malignant lymphomas of diffuse type. They are slightly more common in males. They may arise *de novo* or occasionally as a result of transformation of a pre-existing low grade B cell lymphoma - usually of lymphoplasmacytoid or lymphocytic type.

Typically, the neoplastic immunoblasts are large cells with large round or ovoid nuclei, displaying a prominent, central nucleolus with abundant, pale-staining cytoplasm - generally more than that of a centroblast. In most tumours, some of the cells show plasmacytoid differentiation i.e. they are smaller, have more clumped chromatin, darkly-staining cytoplasm which may show a pale area adjacent to the nucleus corresponding to the position of the golgi apparatus (Figure 1.6).

B immunoblastic lymphomas vary not only in degree of plasmacytoid differentiation but also in cell size. At one end of the range there are tumours composed of small blast type cells whilst at the other end the cells may be almost double that size. In any given tumour, cell size tends to be uniform though exceptions occur and pleomorphic ML Ib-B are sometimes seen containing bizarre giant blast cells. ML Ib-B show a high mitotic rate and atypical mitoses are often seen especially in the pleomorphic sub-type. Spontaneous necrosis is also a common feature accompanied by a heavy infiltration of macrophages and neutrophils (Stansfeld 1992b).

B immunoblastic lymphomas always synthesise immunoglobulin. Monotypic immunoglobulin is found on the surface of the cells and occasionally intra-cellular deposits of immunoglobulin are found in the cytoplasm. In these cases of ML Ib-B showing strongly developed plasmacytoid characteristics, cytoplasmic immunoglobulin is readily shown



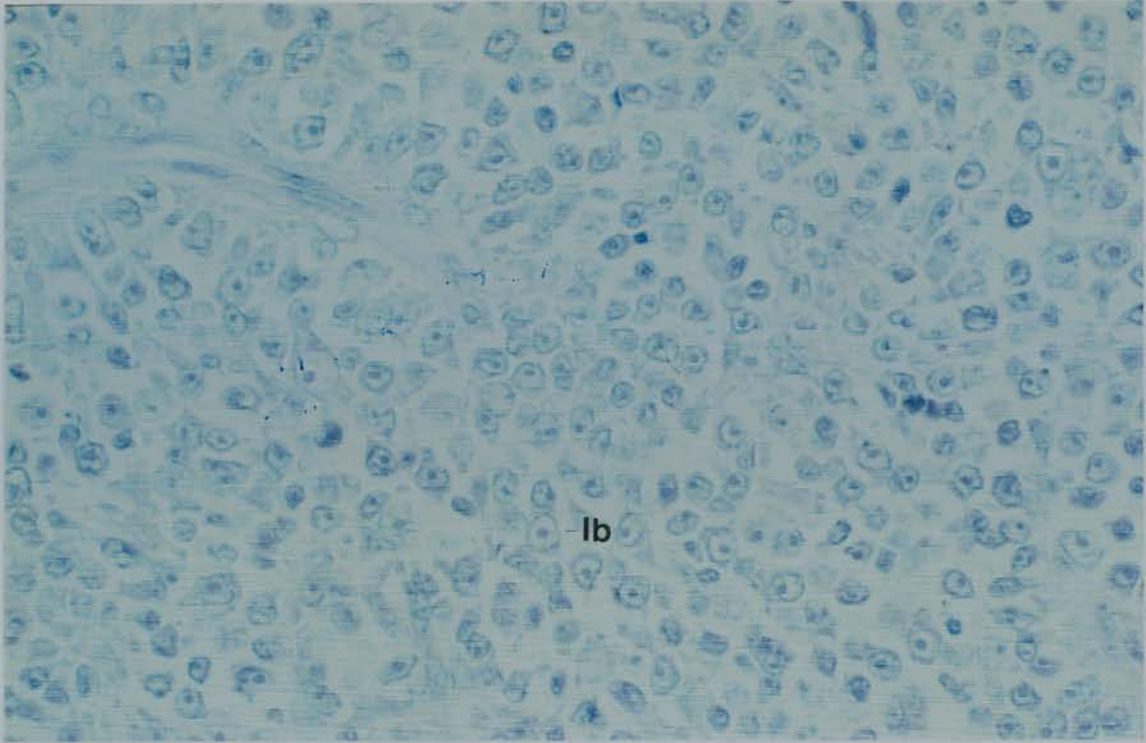
by appropriate immuno-staining. Lennert (1978) states that as a rule, immunoblastic lymphomas reveal a great increase in immunoglobulin in the tumour tissue and/or blood.

### **1.2.8      ML Large Cell Anaplastic Lymphoma (LCAL) or Ki-1 Lymphoma - B Cell Type**

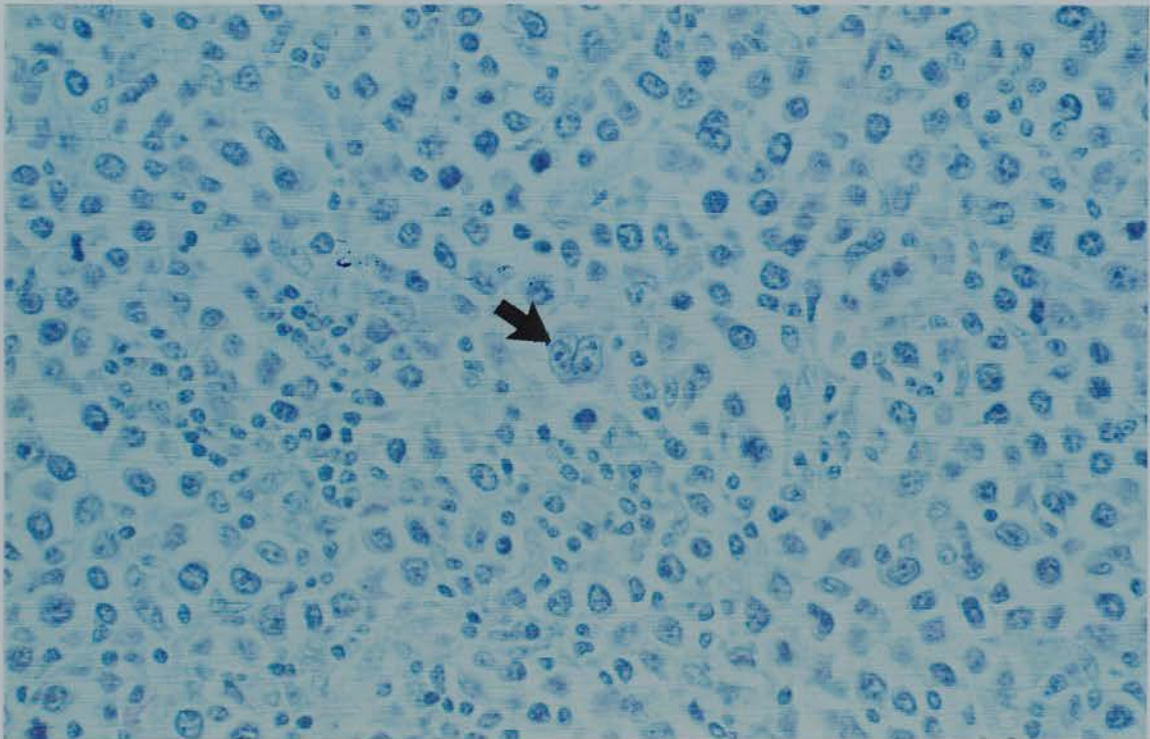
Prior to the recognition of the distinct features of LCAL, many of these lymphomas had been mistakenly diagnosed as metastatic carcinoma, pleomorphic immunoblastic sarcoma, lymphocyte depleted Hodgkin's disease or malignant histiocytosis (Stein et al 1985).

LCAL predominantly involves lymph node or skin (Agnarsson & Kadin 1988). The affected lymph node shows a large cell infiltrate with a prominent sinus distribution that extends from the sub-capsular sinuses into the paracortical region often surrounding residual germinal centres. The tumour cells are very large, pleomorphic and often polyploid cells. The large nuclei are highly irregular and sometimes multiple, nuclear pleomorphism with some folding and lobation is common. Prominent nucleoli are seen, while the cytoplasm is abundant. Mitotic figures are frequent and very often atypical. Tumour giant cells are often present and may be Reed-Sternberg-like although there is generally more nuclear pleomorphism in LCAL than Hodgkin's disease (Glick et al 1976). In addition, small reactive cells such as lymphocytes, macrophages, plasma cells and eosinophils may be present (Figure 1.6).

The tumour cells express the CD30 antigen (Ki-1) uniformly and strongly (Stein et al 1985). Although some LCAL fail to express any T or B



Immunoblastic lymphoma (Case I) comprised mainly of immunoblasts with very few centroblasts. Mag x250



Large Cell Anaplastic Lymphoma (Case L) showing the tumour cells with a giant RS-like cell.-**arrow**

**Figure 1.6** Histological Appearance of Immunoblastic Lymphoma and LCAL (B cell type)

cell markers, immunophenotyping and genotyping studies have shown that the B cell type is less common (Chan et al 1989, Herbst et al 1989). The few cases that show both T cell receptor and immunoglobulin gene rearrangements are interpreted as of B cell origin since 5-10% of B cell lymphomas show T cell receptor gene rearrangements (O'Connor et al 1985). Additional marking has been noted with antibodies against HLA-DR, IL-2 receptor, transferrin receptor and the Ki-67 proliferation marker indicating the activated, proliferative state of the neoplastic cells (Chan et al 1989). Weak leucocyte common antigen (LCA) positivity is usually present (Hall et al 1988) and many may express the epithelial membrane antigen (EMA) (Chan et al 1989).



## **1.3 Aetiology of Hodgkin's and Non-Hodgkin's Lymphomas**

### **1.3.1 Hodgkin's Disease**

The cause of Hodgkin's disease remains unknown. Tilly et al (1991) found abnormal karyotypes found in around 50% of cases. More recently, Poppema et al (1992b) showed abnormal metaphases in 23/28 HD cases. The most common structural abnormalities were in 14q, including the region of the Ig heavy chain, 14q32. By *in situ* hybridisation, cells with numerical clonal abnormalities were shown to be RS/H cells (Poppema et al 1992b). No chromosomal abnormality appears with sufficient frequency to suggest a pathogenic association - karyotyping in Hodgkin's disease is however made difficult by the paucity of the neoplastic RS cells. Recently, the t(2;5)(p23;q35) translocation found in T-cell derived LCAL was found in 11/13 cases of HD of NS and MC sub-types (Orscheschek et al 1995) - however, this was not found in several other studies (Chan et al 1995, Downing et al 1995, Lucey & Shearer 1995, Poppema 1995).

Studies of oncogene involvement in HD have also been hindered by the scarcity of RS cells with studies largely restricted to HD cell lines. Although a number of oncogene products have been found in HD cell lines, including c-myc, p53, c-jun, c-raf, N-ras and others, no characteristic pattern has emerged. Also, the *in vivo* relevance of this is uncertain (Jucker et al 1990). However, recent studies using HD biopsy material have investigated *bcl-2*, *p53* and *c-myc*.

The *bcl-2* proto-oncogene is deregulated via the t (14;18)(q32;q21)

IgH -*bcl-2* translocation seen in 90% of follicular lymphomas (a low grade NHL) and 20% of other high grade NHL. Bcl-2 protein inhibits apoptosis and therefore increases cell survival (Hockenbery et al 1990). Some studies have detected the *bcl-2* translocation in HD (Stetler-Stevenson et al 1990, Lorenzen et al 1992, Reid et al 1993) whereas others have failed to detect the *bcl-2* translocation (Louie et al 1991, Said et al 1991, Athan et al 1992). Immunohistochemical studies have also given rise to conflicting results with some detecting bcl-2 in RS/H cells in a proportion of HD cases (Armstrong et al 1992a, Khan et al 1993, O'Grady et al 1994b) and others failing to find detection of the protein (Algara et al 1991, Louie et al 1991). There is however no correlation between the presence of the bcl-2 protein in RS/H cells and the t(14;18) translocation detectable by PCR (Lorenzen et al 1992). It therefore seems unlikely that *bcl-2* plays a major role in the pathogenesis of HD.

The *c-myc* oncogene is involved in cell proliferation and differentiation and seems to play an important role in cell cycle control. Activation of the *c-myc* proto-oncogene by the t(8;14) *c-myc*-IgH translocation is involved in Burkitt's lymphoma (Dalla-Favera et al 1982). Studies of *c-myc* expression in HD are more limited (Mitani et al 1988, Jiwa et al 1993). However, Jiwa et al found expression of the *c-myc* protein in 94% of cases of HD, restricted to the RS/H cells. Bcl-2 has been found to synergise with *c-myc* in tumour progression (Strasser et al 1990, Fanidi et al 1992) and Jiwa et al (1993) found that in 72% of their HD cases the RS/H cells expressed both bcl-2 and *c-myc* and suggest that the co-operation of bcl-2 and *c-myc* may be involved in the pathogenesis in a substantial number of HD cases.

The *p53* tumour suppressor gene is thought to have a role in the regulation of the normal cell cycle, apoptosis and response to DNA damage

(Lane 1992). Mutation of this gene is thought to be the most frequent abnormality in human tumours. Normal p53 is undetectable by immunohistochemical staining, but stabilisation by mutation or binding to cellular or viral proteins allows detection. P53, restricted to RS/H cells, has been detected in 32 - 74% of HD cases (Doglioni et al 1991, Gupta et al 1992, Niedobitek et al 1993) in all sub-types except LPHD. This suggests that p53 may play a role in some cases of Hodgkin's disease. Recently, Trumper et al (1993) examined one case of HD using single cell PCR technique and detected a mutation in exon 7 of the *p53* gene in 5 out of 7 RS cells.

### **1.3.2      Non-Hodgkin's Lymphomas**

As stated before, Non-Hodgkin's lymphomas represent neoplastic counterparts of reactions which usually take place after antigenic stimulation - each malignant cell type has its benign analogue in the non-neoplastic lymph node (Van der Valk & Meijer 1988). The derailment causing a NHL can be a block in the normal differentiation process or an increased and uncontrolled proliferation. Abnormal chromosomal karyotypes are presumed to occur in 100% of cases (Offit & Chaganti 1991). The most common abnormalities involve translocations (or inversions) at Ig and TcR loci.

Various chromosomal abnormalities have been described for the T cell NHL involving any of the TcR chain genes. Examples are t(8;14)(q24;q12) involving *c-myc* and TcR $\alpha$  (Shima et al 1986) and t(7;9)(q34;q33) involving probably the *c-abl* proto-oncogene and TcR $\beta$  (Hecht et al 1985) amongst many others (Greisser 1989). Some LCAL



cases, especially those of T cell type, have a specific translocation t(2;5)(p23;q35) involving a possible growth-regulating tyrosine kinase (Mason et al 1990).

The most common translocations seen in B cell NHL are the t(8;14)(q24;q32) *c-myc* - IgH which activates the *c-myc* oncogene seen in Burkitt's lymphoma (Dalla-Favera et al 1982) and other high grade B-NHL and the t(14;18)(q32;q21) IgH - *bcl-2* which activates the *bcl-2* oncogene seen in 90% of centrocytic-centroblastic follicular lymphomas and 20% of other high grade B-NHL. Another less common translocation seen in B-NHL is the t(11;14)(q13;q32) *bcl-1* - IgH which activates the *bcl-1* oncogene seen in lymphocytic and centrocytic B-NHLs (Williams et al 1991, Withers et al 1991).

The p53 protein has also been found to be abnormally expressed in a substantial proportion of NHL cases (Pezzella et al 1993).

The incidence of NHL has been rising steadily over the last 30 years in several countries and has increased by over 50% in the USA in the last 6 years (Ries et al 1990). Several lines of evidence have attributed occupational factors such as exposure to pesticides (Zahm & Blair 1992), wood, solvent or related chemicals (Pearce & Bethwaite 1992) or poor nutrition (Davis 1992) and use of hair dye (Pearce & Bethwaite 1992) as having possible aetiological roles.

The most firmly established cause of NHL is immunodeficiency whether due to hereditary or acquired disease (such as AIDS) or to medical treatment (such as post-transplant patients) (Kinlen 1992, Filopovich et al 1992). Mounting evidence has suggested an aetiological role for EBV in these situations (see section 1.4.2).

There is also evidence that EBV may also play a role in both NHL and HD in immunocompetent individuals. Another virus which has been clearly shown to cause NHL is the Human T cell Lymphotropic Virus Type 1 (HTLV-1) (Mueller et al 1991 & 1992). This retrovirus establishes a latent infection in its target cell (activated T helper cells ) and can induce an adult T cell leukaemia or lymphoma (ATL) (Table 1.1).

However, the most established link is between HD and NHL and the Epstein-Barr virus, and because of the role of EBV in the SCID tumours grown in this thesis, the EBV is discussed in more detail in the following section.

## **1.4 The Epstein-Barr Virus**

### **1.4.1 The EBV Genome and Transforming Functions**

The Epstein-Barr Virus (EBV) is a double-stranded DNA Herpes virus that infects all human populations - greater than 95% of the adult human population carries the virus, which is normally acquired asymptotically in early childhood. EBV usually initiates human infection in the oropharyngeal epithelium, which allows viral replication. In this primary infection, EBV infects B lymphocytes which traffic close to the basement membrane of the oropharyngeal epithelium. EBV does not usually replicate in B cells but instead establishes latent infection at a level of about 1 in  $10^5$ - $10^6$  peripheral blood mononuclear cells in healthy adults. It enters B lymphocytes via the C3d receptor (CD21) and immortalises the infected cells. These yield progeny which proliferate indefinitely, maintaining EBV in a latent state (reviewed by Miller 1990, Kief & Liebowitz 1990).

The EBV genome is a linear duplex molecule of approximately 172 kilobase pairs which is not integrated but maintained as a circular molecule in infected cells. The ends of the linear molecule contain direct repeats (terminal repeats) which are joined together on circularisation in the infected cell. EBV has the capacity to encode about 100 genes, but in latent infection only a restricted number are expressed (reviewed by Middleton et al 1991, Rogers et al 1992). 2 of these encode small, nonpolyadenylated non-coding RNAs (EBER1 and 2). In latently infected cells, the EBERs are by far the most abundant gene transcripts ( $10^7$  copies/cell). They are located in the nucleus where they are complexed to cellular proteins. Despite the high abundance of the EBERs, their function is still unknown (Khan et al 1992).

At least 9 virally encoded proteins are expressed during latent infection, these include 6 nuclear proteins - the Epstein-Barr Nuclear Antigens (EBNAs) and 3 Latent membrane Proteins (LMP 1, 2a, 2b). 3 of these viral proteins have been studied in detail - EBNA-1 and 2 and LMP-1. The EBNA-1 protein performs house-keeping functions - it is a DNA-binding protein vital for replication and maintenance of the viral genome in a plasmid form. Expression of LMP and EBNA-2 appear to be essential for the immortalisation of B cells. Although the exact mechanism of the immortalisation process is unclear, EBNA-2 and LMP are known to induce certain activation and adhesion molecules including ICAM-1, LFA-1, LFA-3, CD30, CD39, CD43, CD70, CD23 and CD40 (reviewed by Middleton et al 1991, Rogers et al 1992). CD23 and CD40 are of particular interest as they are both involved in B cell growth and reduction in tumour cell apoptosis, probably through induction of the bcl-2 onco-protein (Liu et al 1991a, Liu et al 1991b). LMP also induces bcl-2 expression and protects the cell from apoptosis (Henderson et al 1991). EBNA-2 and LMP co-operatively induce high levels of CD23 and soluble CD23 is shed from the cell surface where it may act as an autocrine B cell growth factor (Swendeman & Thorley-Lawson 1987, Wang et al 1990).

In B cells, 3 patterns of expression of the latent proteins have been described :- in Type I Latency only the EBNA-1 protein is expressed, in Type II EBNA-1 is expressed along with the LMPs and in Type III all 9 proteins are expressed (Rowe et al 1992). This B cell immortalisation is demonstrated *in vitro* in EBV-transformed B cell lines or Lymphoblastoid Cell Lines (LCL) - cell lines of non-neoplastic, diploid origin grown from seropositive individuals or *in vitro* artificial EBV infection of lymphocytes (reviewed by Tatsumi 1992). These Lymphoblastoid cell lines express the full range of EBV proteins i.e.

Latency Type III. They show high expression of the aforementioned adhesion molecules (causing them to grow in 'clumps') and activation molecules. They show surface and cytoplasmic immunoglobulin, are multi-clonal in nature and show secretion of immunoglobulin (reviewed by Middleton et al 1991, Tatsumi 1992).

#### **1.4.2      Disease Association**

Delayed primary EBV infection results in the development of a transient, benign lymphoproliferative disorder known as Infectious Mononucleosis. EBV is also associated with a range of lymphoid malignancies including nasopharyngeal carcinoma where EBV is regularly found in all cases of undifferentiated and differentiated forms of the carcinoma, and Burkitt's lymphoma (BL) - a high grade B cell lymphoma also involving translocation of the c-myc oncogene (reviewed by Miller 1990). Burkitt's lymphoma shows a Type I latency infection pattern as indeed do BL cell lines, retaining the phenotype of the original tumour. They display very low or undetectable levels of the activation and adhesion molecules that are abundantly expressed on LCLs and instead display other markers (CD10, CD71). The down-regulation of these molecules may provide a mechanism for the tumour to escape immunosurveillance. On serial passage however, there is a tendency for these BL cell lines to 'drift' and acquire an LCL-like phenotype (Rowe et al 1992, Hamilton-Dutoit et al 1993).

A third group of patients in whom EBV-associated tumours have been described is in the immunocompromised population. B cell NHL account for 43% of tumours occurring in primary immunodeficiencies (Filipovich 1984).



Post-transplant patients show a similar high incidence of NHL (although Hodgkin's disease is rare in these patients) (Penn 1984) - these lymphomas almost invariably contain EBV DNA (Crawford et al 1980). Patients with AIDS not only show an increased risk of NHL but also Hodgkin's disease with the great majority of all AIDS -associated lymphomas expressing EBV (Uccini et al 1989, Ioachim et al 1991, Samoszuk & Ravel 1991).

Histologically, the EBV positive lymphomas seen in immunocompromised individuals represent a spectrum of B cell proliferative diseases ranging from polyclonal B cell proliferations (classified as polymorphic diffuse B cell hyperplasia - PDBH) to monoclonal B cell lymphomas (classified as polymorphic B cell lymphoma - PBL). PDBH is characterised by a polymorphic, diffuse, invasive, B cell proliferation involving centroblasts, centrocytes, immunoblasts, plasmacytoid cells and typical plasma cells. The large cells do not show atypia. PBL is distinguished from PDBH primarily by the presence of large immunoblasts with marked nuclear atypia and extensive necrosis. The polyclonal proliferations may evolve to monoclonal in association with appearance of cytogenetic abnormalities (Frizzera et al 1981, Hanto et al 1983). They show a Latency III EBV expression, and similar activation/adhesion molecule expression as LCL (Thomas et al 1990). Recently, it was discovered that in AIDS-related large cell lymphomas all 3 patterns of viral latency could be found - Latency I similar to BL, Latency III reminiscent of LCL and intermediate Latency II (the phenotype seen in Hodgkin's disease) (Hamilton-Dutoit et al 1993).

Lymphomas arising on a back-ground of immunodeficiency differ from lymphomas arising in immunocompetent individuals in that they are frequently extranodal (most commonly presenting in the central nervous



system) and they are more commonly associated with EBV. The debate continues over whether the presence of EBV is secondary to the immunodeficient state of these patients or whether EBV plays a more direct role in oncogenesis.

Recent evidence has accumulated indicating that EBV may play a role in lymphomagenesis in immunocompetent individuals. Recent studies have described the presence of EBV in both Hodgkin's and Non-Hodgkin's lymphomas.

### **1.4.3      Association with Hodgkin's Disease**

EBV has been implicated in Hodgkin's disease both serologically and epidemiologically. Raised antibody titres to EBV were found in Hodgkin's patients compared with other lymphoma patients (Levine et al 1971), and later it was shown that these increased antibody titres occurred before diagnosis of Hodgkin's (Mueller et al 1989). Several studies demonstrated that patients with a history of infectious mononucleosis have an increased incidence of HD compared with control populations (Rosdahl et al 1974).

Recent studies using southern blot analysis have demonstrated the presence of EBV genomes in a proportion (18-41%) of Hodgkin's disease cases (Weiss et al 1987, Anagnostopolous et al 1989, Gledhill et al 1991, Jarrett et al 1991).

Infectious EBV virions contain linear double-stranded DNA with direct terminal repeats (TR) of about 500 base pairs. The number of TRs at each end of the viral DNA is variable. However, after infection of a cell, the termini are joined to form a circular episome. If the multiplicity of the

infection is not more than 1 (i.e. if that cell undergoes clonal expansion) all the episomes within a cell and the progeny of that cell will have the same number of TRs. This is assessed using a probe for the viral terminal repeats, if monoclonal - one band only appears on the blot (Raab-Traub & Flynn 1986).

Analysis of the terminal repeats of the EBV genome in these studies has shown that the infected cells in the HD biopsies are clonal with respect to EBV - consistent with the expansion of a single EBV-infected cell.

In addition, some studies have demonstrated the localisation of the EBV to the RS/H cells in the HD biopsies using *in situ* hybridisation (for EBV EBER RNA) and immunohistochemical studies (Brousset et al 1991, Weiss et al 1991, Armstrong et al 1992b, Delsol et al 1992). Using the EBER *in situ* hybridisation, it was shown that in a proportion of HD cases with EBV-infected RS/H cells, an additional infection of very few small non-malignant lymphoid bystander cells was seen. These cells were small lymphocytes - no EBER positive blasts were seen. These EBV positive bystander cells in the HD biopsies were found to correspond qualitatively with the occasional EBV-infected cell in normal lymphoid tissue. Immunohistochemical studies however showed that LMP expression was constantly restricted to the RS/H cells, the EBER positive bystander cells being consistently negative for LMP. It appears therefore that the EBER *in situ* hybridisation and LMP immunostaining methods are more suitable for the determination of the EBV status of Hodgkin's compared to non-morphological methods such as PCR which cannot discriminate between cases containing infected tumour cells and those cases only containing infected bystander cells (Hummel et al 1992, Khan et al 1992).

The expression of LMP and EBNA-2 has been investigated using immunohistochemistry. The LMP-1 protein is expressed in RS/H cells in EBV positive cases but EBNA-2 has not been detected (Pallenson et al 1991a, Armstrong et al 1992b, Delsol et al 1992). A study on EBV latent gene expression at the RNA level showed the presence of EBNA-1, LMP-1 and LMP-2 transcripts but absence of EBNA-2 mRNA (Deacon et al 1993) - i.e. a Latency II infection pattern. EBV lytic cycle proteins have been detected in only occasional RS/H cells in a minority of cases of Hodgkin's (Pallenson et al 1991b). The expression of LMP by RS/H cells suggests that EBV is playing some role in the pathogenesis of Hodgkin's disease.

LMP is known to induce certain cellular genes such as CD23, CD40 and bcl-2 (Section 1.4.1). CD23 (minority of cases ) and CD40 (majority of cases) have been detected on RS/H cells (Herbst et al 1991, Armstrong et al 1992a, O'Grady et al 1994b). Some studies have detected the bcl-2 protein in RS/H cells in a proportion of HD cases (Armstrong et al 1992a, Khan et al 1993, O'Grady et al 1994b) whereas others have failed to find such expression of bcl-2 (Algara et al 1991, Louie et al 1991). However, no clear correlation between LMP-1 expression and detection of these proteins in RS/H cells was found (Armstrong et al 1992a, Khan et al 1993, O'Grady et al 1994b). Therefore the role of LMP-1 in Hodgkin's disease remains unresolved but it appears to be independent of the upregulation of CD23, CD40 and bcl-2.

There is also no evidence that LMP affects the eventual prognosis of the associated cases (Vestlev et al 1992). It is possible that LMP-1 expression induces a cytotoxic T cell response against the host RS/H cells (Murray et al 1988) which counterbalances any growth advantage provided by the protein. The fact that a high frequency of LMP-1 positive cases of HD

are found in AIDS patients (Audouin et al 1992) lends some support to this possibility.

It is clear that EBV is only associated with a proportion (33-50%) of cases. This does not detract from the importance of the viral association as the pathological and epidemiological features of Hodgkin's disease suggest that it is a heterogeneous condition likely to have more than one aetiology (Macmohen 1966). Epidemiologically, HD can be divided into 3 main groups corresponding to the age groups 0-14 years, 15-34 years and 50 years and over. NSHD largely accounts for the peak incidence in the young adult age group whereas MCHD is relatively more common in child-hood and older adults (Alexander et al 1991). Risk factors for the development of HD differs in the different age groups - young adult HD appears to be associated with a high standard of living suggesting that a delayed exposure to a common infectious agent may be involved (Macmohen 1966, Gutesohn 1982, Alexander et al 1991).

EBV has been detected in the RS /H cells of all sub-types of HD. However, Pallenson et al (1991a) found a significant increase in LMP expression in the MC sub-type. This was subsequently confirmed in some (Staal et al 1989, Weiss et al 1991, Delsol et al 1992, Murray et al 1992, Weinreb et al 1992, O'Grady et al 1994a) but not all (Herbst et al 1991, Jarrett et al 1991) studies.

Jarrett et al (1991) also reported an excess of EBV positive cases in the paediatric (0-15 years) and older adults (50 years and over) age groups compared to the young adult cases. Some, but not all studies have confirmed this age distribution (Libetta et al 1990, Herbst et al 1990, Coates et al 1991, Vestlev et al 1992, Weinreb et al 1992). The results of the Jarrett et al (1991) study supports the hypothesis that HD involves different

aetiologies in the different age groups with EBV involved in the pathogenesis of paediatric and older adult cases (possibly explaining the higher incidence of EBV positive MCHD cases). They speculate that *de novo* EBV infection is important in the paediatric group with a reactivation of latent infection, possibly as a result of declining T cell immunity occurring in the older age group.

The low level of EBV positivity in the young adult age group (the major sub-type being NSHD) suggests that other factors are involved in these cases. This is interesting since this was the group originally most thought to be associated with an infectious agent on epidemiological grounds (MacMohen 1966, Gutesohn 1982, Alexander et al 1991). Clustering of HD cases is also most likely to occur in cases aged under 35 years and in the Nodular Sclerosing sub-type (Alexander et al 1989, Alexander et al 1991).

Therefore it is possible that another virus may be involved in the pathogenesis of HD in the young adult group - viruses studied include the herpesviruses HHV-6 and HHV-7 and cytomegalovirus (CMV). Serological studies have implicated HHV-6 as HD patients, particularly those in the young adult group, were found to have elevated antibody titres to HHV-6 antigens (Clark et al 1990). However, several groups have been unable to detect HHV-6 or 7 in any HD cases using southern blotting or DNA *insitu* hybridisation (Gledhill et al 1991, Khan et al 1993, Jarrett et al unpublished results). One study was able to detect HHV-6 in 2 cases using southern blotting (Torelli et al 1991). Studies on CMV have also given negative results (Weiss et al 1987, Khan et al 1993)

O'Grady et al (1994a) investigated whether EBV in Hodgkin's disease was related to the site of origin of the tumour. For Stage I Hodgkin's, LMP-1



expression by RS/H cells was significantly associated with presentation in neck nodes compared with non-neck nodes. (No correlation was seen in Stages II - IV disease possibly because biopsy site does not necessarily indicate site of origin in these stages of disease).

Therefore, detection of EBV in HD may be related to presentation in nodes draining the oropharyngeal lympho-epithelial tissue - the main site of EBV proliferation after infection. In infectious mononucleosis, the tonsils and cervical lymph nodes, but not the spleen or inguinal lymph nodes contains many strongly LMP-1 positive RS-like cells (Isaacson et al 1992) and other EBV-associated tumours such as Burkitt's lymphoma and Nasopharyngeal carcinoma tend to arise in the head and neck region. The cervical lymph nodes are one of the commonest sites of origin in HD with axillary and mediastinal less common.

At present, the role of EBV in HD as a pathogen or passenger is not clear. However, the localisation of EBV to the RS/H cells and the clonality of the EBV episomes in HD suggests a pathological role for EBV in at least some cases (30-50%). Other aetiological factors may be important in the young adult (predominantly NSHD) sub-group.

#### **1.4.4      Association with Non-Hodgkin's Lymphoma**

Although there is very strong evidence that EBV is causally associated with the occurrence of NHL under conditions of acquired or inherited immune suppression, the evidence is much less clear in relation to the risk of NHL in apparently non-immunocompromised individuals.



However, there has been a number of recent reports of EBV in peripheral T-NHL in immunocompetent individuals (Su et al 1990, Su et al 1991). The EBV genome appeared to be clonotypically proliferated in the neoplastic T cells using immunocytochemistry. Hamilton-Dutoit & Pallenson (1992) also found a frequent association of EBV with peripheral T cell lymphomas (10%), but surprisingly not as frequent in B-NHL (4%) - these findings are in keeping with previous reports (Weiss et al 1987, Staal et al 1989, Sabourin et al 1993). The majority of these tumours displayed a Latency Type II pattern of infection (LMP positive, EBNA-2 negative). Ott et al (1993) found a higher incidence of EBV DNA in B-NHL (10% of cases) however, this was still lower than in the T-NHL (33% of cases) in this study.

This may be surprising given the B cell tropism of EBV. However, increasing evidence from both *in vitro* and *in vivo* studies suggests that EBV can infect T lymphocytes. An EBV receptor-like molecule has been demonstrated *in vitro* on malignant T cell lines (Fingerth et al 1988) and T lymphocytes transfected *in vitro* with EBV DNA become immortalised (Stevenson et al 1987). The route of entry of EBV into T cells and the role of EBV in the pathogenesis of T cell lymphoma is yet to be established, but the finding of clonotypic EBV in these tumours (Su et al 1991) suggests an oncogenic role for EBV in these T cell lymphomas.

A high incidence of the EBV genome has also been detected in the CD30 (Ki-1) positive NHL, including Large Cell Anaplastic Lymphomas (LCAL) (Ross et al 1992) and non-LCAL (with 5 - 35% CD30 positive cells) (Kanavaros et al 1992). 29/38 CD30 positive B-NHL, 9 of 20 CD30 positive T-NHL and 2 CD30 positive NHL of undetermined lineage were found to be EBV positive by PCR. Of 29 CD30 negative NHL, only 6 were EBV positive

(5 B-NHL and 1 T-NHL). *In situ* hybridisation demonstrated the EBV genome exclusively in the nuclei of the tumour cells in a proportion of the EBV positive cases. Immunostaining again showed a Type II latency pattern of infection, but LMP expression was only found in the CD30 positive NHL.

Although the biological modulation of this relationship is unclear, the authors hypothesise that the presence of CD30 positive tumour cells in NHLs increases the probability that these tumours harbour EBV and this may have clinical significance since it is suggested that the presence of EBV in peripheral T cell NHL is associated with a more aggressive course (Su et al 1991).

Mueller et al (1992) found evidence that among a group of 104 NHL patients with blood samples taken several years before diagnosis, there was an elevated antibody titre against EBV, which is quite similar to that seen in immuno-suppressed patients prior to diagnosis - this pattern was most evident in older patients. This suggests that EBV-associated NHL is more common in older persons and may be related to more chronic viral activation due to subclinical immune suppression due to ageing.

## **1.5 Previous Models of Hodgkin's Disease and Non-Hodgkin's Lymphoma**

### **1.5.1 Cell Lines**

#### **Hodgkin's Lymphoma Cell Lines**

The failure to define the cellular origin of Hodgkin's Disease is largely due to the small number of neoplastic RS/H cells found within biopsy material. Consequently, this causes difficulty in distinguishing the immunophenotype or genotype of the neoplastic cells from the surrounding normal lymphoid and histiocytic cells. There have been several attempts to purify RS/H cells *in vitro*. Sitar et al (1989) attempted this using isopyknic centrifugation and velocity sedimentation, this achieved a substantial purification. However this approach has rarely been successful. *In vitro* cultivation techniques have been used in attempts to enrich and purify RS/H cells in temporary or permanent cultures. Development of HD cell lines has proven to be more difficult than NHL cell lines. Only a few cell lines containing cells that resemble RS/H cells have been established as the *in vitro* culture conditions favour the out-growth of "contaminating" residual normal bystander cells such as EBV-transformed B lymphoblastoid cells, fibroblasts, or monocyte/macrophage monolayers. There is a striking heterogeneity of morphological variations among the cell lines, but also within the population of each cell line. Some cell lines express one or more markers associated with T cells (CO, HDLM-2, HO, L-540), B cells (DEV, HD-70, KM-H2, L-591, SUP-HD1) or myelomonocytic cell lineages (SU, RH-HD-1). 2 of the cell lines (ZO, L-428) appear to be devoid of cell lineage-associated markers. The results of gene rearrangements studies are also heterogeneous with

some cell lines showing TcR gene rearrangements (CO, HDLM-2, HO, L-540), Ig gene rearrangements (DEV, HD-70, KM-H2, L-591, ZO) and both (L-428, SUP-HD1) (reviewed by Drexler 1993).

The relationship of the Hodgkin cell lines to the RS/H cell remains to be established - the artifactual expansion of non RS/H cells in culture cannot be excluded. New strategies for the investigation of HD and the histogenesis of the RS/H cell are therefore necessary.

#### Non-Hodgkin's Lymphoma Cell Lines

There are many established NHL cell lines. However, these cell lines are established only by chance and are not applicable to a large number of cases. NHL tumour cells have been found to be difficult to grow *in vitro* with the exception of Burkitt's lymphoma possibly because of the involvement of the Epstein-Barr virus. Most B cell lines are derived from BL, lymphoblastic lymphoma or large cell lymphoma, although an immunoblastic cell line was first established recently (Ho et al 1990). NHL cells do not usually grow spontaneously *in vitro*, under standard *in vitro* culture conditions, and the use of an exogenous lineage-specific growth factor (B cell growth factor) was necessary to establish NHL-B cell lines (Ford et al 1990). Although many lines have been shown to be useful in studying the characteristics and molecular biology of NHL (Mohamed & Al-Katib 1988, Saltman et al 1988, Dyer et al 1990), such cell lines occasionally show characteristics different from those of the original tumour because of tumour progression *in vitro*.

Therefore, a satisfactory *in vivo* animal model of NHL would be more suitable to enable a closer correlation with the *in vivo* situation of the human disease.



### **1.5.2 Nude Mice**

These are also known as *nu/nu* mice. These are mice with congenital absence of the thymus, and whose blood and thymus dependent areas of the lymph nodes and spleen are depleted of T lymphocytes. These mice are homozygous for the gene 'nude', having no body hair.

#### **Hodgkin's Disease**

Neither HD-derived cell lines nor primary biopsy material could be grown in nude mice (Epstein et al 1976, Watanabe et al 1980, von Kalle et al 1992). Therefore it has not been possible to study the *in vivo* growth characteristics of HD using nude mice. Growth of HD cell lines in nude mice was limited to intracranial growth of one HD-derived cell line (Engert et al 1987) and intracranial growth of EBV positive polyclonal cells from a HD biopsy specimen (Schaadt et al 1979). It was necessary to use the intracranial route of transplantation because the brain is an immunologically privileged site, thus allowing better tumour take rates and latency periods.

#### **Non-Hodgkin's Lymphoma**

Growth of NHL biopsy material has also been attempted in nude mice. Most trials using this model failed - for example, Kuga et al (1975) were unable to maintain any of 12 biopsy specimens transplanted in nude mice. Later, additional treatments were used to increase the chance of success. Epstein et al (1976) used intracranial injection and succeeded in growing 8/14 NHL biopsy specimens in nude mice. Watanabe et al (1980) used irradiation with and without splenectomy and achieved a 29% growth rate. However, the

majority of the cases required irradiation and splenectomy for growth to occur. The most recent study using nude mice was that of Igarashi et al (1989) who used total body irradiation as pre-treatment. 27% (6/22) of the transplanted lymphomas were established as nude mouse lines, the transplanted lymphomas substantially retaining the characteristics of the original lymphomas. However, only lymphomas of advanced clinical stage (one was of Stage III, the remaining 5 of Stage IV) grew in the nude mice.

The problem with the nude mouse model is the pre-treatment necessary for lymphoma growth to occur. Also, these early studies did not consider the possibility of the involvement of the EBV in these nude mouse tumours, and it is possible that some were simply EBV-driven lymphoproliferations (all of the nude mouse tumours grown by Igarashi et al were found to be diploid).

Therefore, the nude mouse has been shown to be an unsatisfactory *in vivo* model for the study of HD and a very limited model for NHL requiring extensive pre-treatment.

Consequently, new strategies are necessary for the study of HD and NHL, and the more immune-deficient SCID mouse model was used in this thesis in an attempt to establish a satisfactory *in vivo* model to study HD and NHL. The SCID mouse is described in more detail in the following section.

## 1.6 The SCID Mouse

### 1.6.1 Biology, Immunology and Genetic Deficiency

The mutant C.B-17 SCID (Severe Combined Immune Deficient) mouse strain has a heritable, recessive gene defect on chromosome 16 involving the V-D-J recombinase system. V-D-J recombination is mediated by a site specific recombinase that recognises short DNA signal sequences (heptamer/nonamer sequences with either a 12 or 23 base pair spacer) which lie adjacent to each coding V, D and J gene segment. The recombination of any 2 coding elements is thought to involve double-stranded DNA breaks at the coding/signal borders followed by formation of a signal joint and coding joint. In immature SCID lymphocytes this reaction is initiated with signal joint formation, however coding joint formation is reduced 5000 fold. This prevents correct TcR and Ig gene rearrangements and results in a virtually complete functional T and B cell deficiency (Lieber et al 1988 & 1989, Bosma 1989, Harrington et al 1992).

Some developing SCID lymphocytes succeed in forming V-D-J and V-J coding joints, in fact oligoclonal B and T cells have been detected in a low percentage of young adult mice (5-20%) and in virtually all old SCID mice ( $\geq 1$  year old). This "leaky" phenotype is possibly resulting from low efficiency V-D-J recombinase activity or a reversion of the *scid* mutation. (Osmond et al 1992, Hendrickson 1993, Kotloff et al 1993). However, the *scid* mutation has very recently been bred into 2 additional murine strains. SCID mice have been crossed with mice homozygous for the bieve mutation (SCID.BG mice) and the C3H strain (C3H SCID) and this has severely depressed the leakiness of the *scid* mutation (Hendrickson 1993).

SCID mice are deficient for both B and T cell function, however early precursor B cells (showing abnormal V-D-J rearrangements) have been detected in the bone marrow (Osmond et al 1992). Also, most SCID mice show the Thy-1 marker on the majority of the recovered cells from the thymus. SCID mice are lymphopenic and their lymphoid organs are 1/10th or less of normal size. They show a rudimentary thymic medulla with no lymphocytic cortex, splenic follicles and lymph nodes virtually devoid of lymphoid cells and under-developed bronchial and gastrointestinal lymphocytic foci - Peyer's patches are rarely visible in these mice. However the bone marrow is within the norm, cells of the myeloid lineage appear normal and remain active throughout life. They also have normal Natural Killer cells, antigen presenting cells and splenic colony stem cells (Dorshkind et al 1985).

Spontaneous T cell lymphomas occur in  $\geq 10\%$  of SCID mice. They appear to arise in the thymus and are highly invasive and transplantable. They are thought to be a direct or indirect consequence of the *scid* mutation as they are rarely, if ever, observed in normal C.B-17 or BALB/c mice (Bosma et al 1983, Custer et al 1985).

### **1.6.2      The SCID Mouse as an Animal Model System for Studying Human Disease**

The SCID mouse offers several advantages over the previously used athymic T cell deficient nude mouse as an animal model system. The immune deficiency is more complete and there is therefore no need for further immunosuppression such as whole body irradiation used with nude mice.

They are also easier to breed and less expensive and indeed have been shown to allow better growth of human material (Philips et al 1989) and support growth of normal, non-transformed cells, not possible with the nude mouse.

An example of this is the successful transfer of a functional human immune system in the SCID mouse providing an *in vivo* model for a variety of immunologic studies (Mosier et al 1988, Croy 1993, McKerrow & Ritter 1993). The SCID mouse has also been used to study a number of human diseases. A variety of autoimmune diseases have been induced in SCID mice, including systemic lupus erythematosus (SLE), human autoimmune thyroid disease (AITD), Graves disease and rheumatoid arthritis (Segal et al 1992, Taylor 1992). The SCID mouse has also provided a useful animal model for human myeloid, lymphoblastic and lymphocytic leukaemias (Cesano et al 1992, Kobayashi et al 1992) and has served as a relevant *in vivo* model for malignant melanoma and lung tumours (Bankert et al 1989, Taylor 1992) amongst others.

### **1.6.3      Previous Lymphoma Studies**

#### **EBV-associated Lymphoproliferative Disease**

The SCID mouse has been used in many studies as an *in vivo* model to study the pathogenesis of the EBV positive lymphomas seen in immunocompromised individuals such as AIDS or post-transplant patients. This is achieved by intraperitoneal injection of SCID mice with peripheral blood lymphocytes from EBV seropositive donors or from EBV seronegative donors followed by injection of EBV (Cannon et al 1990, Rowe et al 1991,



Mosier et al 1992, Nadal et al 1992, Purtilo et al 1992, Veronese et al 1994) This results in the development of fatal, high grade, EBV positive B cell tumours occurring as abdominal solid tumours closely associated with the liver; additional tumours were also found in the thymus or spleen. These tumours showed a similar histological appearance (large B cell immunoblastic lymphomas with plasmacytoid differentiation and well-defined areas of necrosis), cell surface phenotype and EBV latent gene expression (Latency III) to the large cell lymphomas seen in immunosuppressed patients. They were found to be either polyclonal or oligoclonal which could present at multiple sites as different poly/oligoclonal foci, although monoclonal foci were also detected in some cases. These tumours were found to exhibit the normal diploid karyotype with no abnormal translocations such as bcl-2 or c-myc and were found to show secretion of high levels of immunoglobulin.

### Hodgkin's Disease

SCID mice have been found to support growth of Hodgkin's disease cell lines. All 6 of the cell lines injected - L428, L591, DEV, HD-LM2, KM-H2 and sub-line L540Cy grew in the SCID mice retaining the karyotype and expression of T or B cell markers of the cell lines before inoculation (von kalle et al 1992). However, expression of the EBV was not investigated.

During this project, Kapp et al (1993) also transplanted fresh Hodgkin's disease-derived biopsy tissue into SCID mice. 3/13 cases gave rise to human EBV positive B cell lymphomas. These Hodgkin's disease-derived tumours are discussed in more detail in the General Discussion - Chapter 8 of this thesis.

## Non Hodgkin's Lymphoma

During this project, Itoh et al (1993) also transplanted NHL into SCID mice. SCID tumours developed from 23 of 50 NHL specimens. Of these tumours, 10 were identical to the original NHL tumour but in 11 cases, unrelated EBV positive lymphoproliferations developed and 2 cases showed a mixture of original tumour clones and new EBV positive proliferations. These SCID tumours will be discussed in more detail in the General Discussion - Chapter 8 of this thesis.

Therefore, despite many phenotypic and genotypic studies, the histogenesis of the Reed-Sternberg cell in Hodgkin's disease remains unknown. Neither *in vitro* cell lines nor the nude mouse *in vivo* model appear to be satisfactory to study Hodgkin's disease. Consequently, in an attempt to study the biology of Hodgkin's disease and to investigate the histogenesis of the Reed-Sternberg cell, transplantation of fresh HD biopsy material into SCID mice was carried out in this thesis.

In addition, fresh NHL biopsy material was injected into SCID mice in an attempt to establish a satisfactory *in vivo* model to study the disease without the need for further immunosuppression such as whole body irradiation required in the nude mouse model.

## **Chapter Two**

### **Materials and Methods**

All chemicals were bought from Sigma Immunochemicals UK Ltd. unless otherwise stated.

#### **2.1 SCID Mice**

The SCID mice used in this project were originally bought from the National Institute for Medical Research, Mill hill, London. They were maintained as a stock breeding colony by the staff of the Department of Medical Microbiology Animal House by sister/brother matings. The off-spring were then again selected for a sister/brother mating. The mice were housed in cages of 4 in a half-suit isolator. The isolator was kept sterile by changing the filters once a week and the large Hepa filter every year. All food was irradiated and vacuum-sealed and all bedding and housing was autoclaved. These were all sprayed with disinfectant and left in the port-hole for 20 minutes prior to use in the isolator.

To prevent infections, mice were checked twice a year for Sendai virus, M.Pulmonis virus, and Rodent Coronavirus using an Immunocomb. If an infection did occur in the isolator, all the mice were removed to another isolator. The infected isolator was then fumigated for 24 hours with peracetic acid. Only new mice were then housed in that isolator, the potentially infected mice were not returned.

Screening for "leaky" SCID mice was carried out by ELISA as in Section 2.10

## **2.2 Collection of Lymphoma Biopsies**

Fresh Hodgkin's Disease and Non-Hodgkin's Lymphoma lymph nodes and one spleen were obtained as routine biopsies from the Royal Infirmary and the Western General Hospital, Edinburgh. They were trimmed under sterile conditions and a small piece kept in sterile RPMI medium (Gibco BRL) for use in this project.

## **2.3 Preparation of Tumour Cell Suspension and Injection into SCID Mice**

Initially, cell suspensions of Hodgkin's Disease lymph node biopsies previously frozen down in liquid nitrogen were injected into the SCID mice. No human tumour was grown from any of these cases (10 injected in total) and therefore it was decided to only inject fresh biopsy material.

After removal of the capsule and fatty tissue, the fresh lymph node/spleen was cut into small fragments in RPMI/L-Glut/Pen/Strep (all Gibco BRL) and filtered through a fine mesh strainer. The lymph node cell suspensions were simply centrifuged for 5 minutes at 1000 rpm and resuspended in 1ml of RPMI. The cell number present per ml of suspension was counted in white blood cell counting fluid using a Neubauer Counting Chamber.

Studies were made to determine the optimum method of transplantation. The intraperitoneal mode of injection was found to be the most convenient and gave rise to the greatest number of tumours. 250 $\mu$ l of tumour cell suspension (containing  $0.7 - 7 \times 10^7$  cells) was injected into the peritoneal cavity of each of 4 mice. Injections were performed under sterile conditions in the half-suit isolator.

## **2.4 Mouse Post-Mortem**

The mice were checked twice a week for signs of illness by the staff of the Animal House. Mice were sacrificed when there was detectable tumour (usually abdominal swelling) or poor condition - this included weight loss, lethargy, hunched appearance and poor coat condition. Mice were killed using either ether or halothane inhalation.

Mice were dissected using sterile instruments, and a sample of aortic blood was taken. The size and general appearance of the tumour (if present), the abdominal organs and any visible lymph nodes present were noted. The thoracic organs were checked for the presence of a thymic remnant or a murine thymic lymphoma.

The main bulk of tumour was dissected out and blocks were taken for each of the following:-

- (a) A small piece was placed in sterile PBS to passage the tumour into a further set of mice.
- (b) A piece was snap frozen in OCT for frozen section immunophenotyping.



- (c) A block was snap-frozen for DNA extraction and molecular analyses (see Appendix C).
- (d) Tumour and additional representative blocks of mouse organs including heart and lungs, liver, gut, pancreas, spleen, kidneys, thymus and lymph nodes were placed in 10% neutral buffered formalin (Lothian Chemical Co.) for paraffin section immunotyping.

## **2.5 Passage of Tumour**

The piece of tumour in sterile PBS (Oxoid Unipath Ltd.) was made into a cell suspension in RPMI/L-glut/Pen/Strep as before (2.3) and injected intraperitoneally into a further 4 mice.

## **2.6 Tissue Processing For Paraffin Sections**

All material used for paraffin-embedded tissue sections was fixed in neutral buffered formalin solution. This fixes by reacting primarily with basic amino acids to form cross-linking 'methylene bridges'.

The various SCID organs removed at post-mortem were left to fix in the formalin over-night. The tissues were then trimmed and clipped up in labelled Tissue-Tek cassettes (Bayer Diagnostics) to be processed through a Vacuum Impregnation Processor (VIP)(Bayer Diagnostics). The tissues in the VIP were gently de-hydrated starting in 50% alcohol and slowly increasing (80, 95, 99%) to absolute alcohol over a 14 hour schedule. They were taken through xylene (Genta Medical) to paraffin wax, in which they

were blocked up using a Tissue-Tek Embedding Console (Bayer Diagnostics).

## **2.7 Preparation of Tissue Sections**

### **2.7.1 Cryostat Sections For Immunocytochemistry**

Previously snap-frozen material was partially thawed and transferred onto a metal chuck. This was frozen in OCT (Bayer Diagnostics) onto the chuck using liquid nitrogen or dry ice. 3µm sections were cut at -20°C using a cryostat onto glass slides previously coated with poly-L-lysine tissue adhesive. These were left to dry at room temperature for at least 15 minutes and then fixed in acetone (Genta Medical) for 10 minutes. These slides were wrapped in foil and stored at -20°C until use.

### **2.7.2 Paraffin Sections For Immunocytochemistry**

3µm paraffin sections were cut from ice-cooled paraffin blocks using a microtome and floated on warm water onto glass microscope slides. The slides were then dried over-night in a 55°C incubator. One of the antibodies used in this study (anti-LMP antibody) required that the paraffin sections were floated onto poly-L-lysine coated slides and dried over-night at room temperature.

### **2.7.3 Paraffin Sections For *in situ* Hybridisation**

To ensure that the tissue sections did not 'lift off' the slides during the lengthy *in situ* hybridisation technique, they were floated onto slides pre-coated with the strong tissue adhesive Tespa (3-aminopropyltriethoxysilane).

Also, to prevent RNase contamination, gloves were worn when cutting and sections were floated onto distilled water. The sections were then dried over-night at 37°C, wrapped in foil until use and only handled with gloves.

#### **2.7.4 Paraffin Sections For Flow Cytometry**

50µm paraffin sections were cut for use in flow cytometry. These were dried over-night in a high temperature (70°C) air-dryer before use.

### **2.8 Immunocytochemistry**

Paraffin and frozen section immuno-phenotyping was carried out using a panel of B cell, T cell, monocyte and EBV markers - these are detailed in Tables 2.1 and 2.2.

#### **2.8.1 Assessment of Antibodies**

All antibodies were titrated on appropriate control tissues to determine optimum dilution before use on the SCID tumours. Antigen retrieval techniques were required with some antibodies used.

Many epitopes are sensitive to heat, therefore in several cases sections for use were cut onto poly-L-lysine coated slides and dried over-night at room temperature instead of high temperature drying. For some antibodies, optimal incubation with primary antibody was found to be over-night at 4°C as opposed to the conventional 30 minutes at room temperature. Antibodies for use with formalin-fixed paraffin sections were always titrated with and without trypsinisation. Some antigens are not well demonstrated

after formalin fixation as the formaldehyde may react with the amino acids next to the epitope under investigation causing conformational changes. However, in most cases this can be reversed by pre-digesting the sections with trypsin which allows hidden determinants to be exposed. Trypsin actually cleaves adjacent to lysine and arginine, the 2 amino acids most likely to react with formaldehyde.

### **2.8.2 ABC Method**

This method was used for all immuno-staining of paraffin sections of SCID and original biopsy material. In each run of immunocytochemistry, a known positive control was taken through. This was also run as a negative control by omitting the primary antibody and replacing this with normal serum.

A standard ABC method was used - the tissue sections were deparaffinised in xylene and rehydrated in descending grades of alcohol. Endogenous peroxidase activity was blocked by immersing the slides in 1% hydrogen peroxide for 10 minutes. If necessary (Table 2.1), the sections were then treated with pre-heated 0.1% trypsin (ICN Biochemicals) in 0.1% calcium chloride pH 7.8 at 37°C for 25 minutes.

The sections were then washed in Tris Buffered Saline (TBS) (Fisons) pH 7.6 for 5 minutes before blocking of non-specific binding by incubation in normal serum - normal rabbit serum (Sapu) if using a mouse monoclonal primary antibody and normal swine serum (Seralab) if using a rabbit polyclonal primary antibody. The slides were also washed between each stage with TBS. A 30 minute incubation with primary and biotinylated secondary antibody (Dakopatts) was carried out prior to the AB Complex step. In the later stages of this project, the AB Complex (Dakopatts) was replaced by horse-radish peroxidase conjugated avidin (Dakopatts) on the

**Table 2.1    Details of Antibodies Used on Tissue Paraffin Sections**

ANTIBODY	ANTIGENIC SPECIFICITY	Ig SUB-TYPE	DILUTION	SOURCE
D/LCA	CD45	IgG1	1:20	Dako
L26	CD20	IgG2a	1:1000	Dako
MB1	pan B cell	IgG1	1:40	BNS
MB2	pan B cell	IgG1	1:20 T	BNS
BU38	CD23	IgG1	1:100 T	Binding Site
B-B20	CD40	IgG1	1:50 T	Serotec
F8-11-13	CD45RA	IgG1	1:500	Line
Kappa	Ig kappa light chain	polyclonal	1:500 T	Dako
Lambda	Ig lambda light chain	polyclonal	1:1500 T	Dako
IgM	Ig $\mu$ heavy chain	polyclonal	1:2000 T	Dako
IgG	Ig $\gamma$ heavy chain	polyclonal	1:5000 T	Dako
IgA	Ig $\alpha$ heavy chain	polyclonal	1:2000 T	Dako
T3	CD3	polyclonal	1:100 T	Dako
UCHL1	CD45Ro	IgG2a	1:500	Line
MT1	CD43	IgG1	1:10	BNS
LN3	HLA-DR	IgG2a	1:20	Clonlab
KP1	CD68	IgG1	1:10 T	Dako
D-M1	CD15	IgG1	1:20 T	Dako
Ber-H2	CD30	IgG1	1:10 T	Dako
E 29	EMA	IgG2a	1:40	Dako
CS1-4	LMP	IgG1	1:50	Dako
PE-2	EBNA-2	IgG1	1:10	Dako

**KEY**

T - requires trypsinisation



**Table 2.2    Details of Antibodies Used on Tissue Cryostat Sections**

ANTIBODY	ANTIGENIC SPECIFICITY	Ig SUB-TYPE	DILUTION	SOURCE
D/T3	CD3	IgG1	1:10	Dako
D/T4	CD4	IgG1	1:10	Dako
D/T8	CD8	IgG1	1:10	Dako
OKM1	CD11b	IgG2b	1:10	Ortho
LFA1B	CD18	IgG1	1:10	Dako
D/IL2	CD25	IgG1	1:10	Dako
UCHM1	CD14	IgG2a	1:10	Sapu
HD37	CD19	IgG1	1:10	Dako
TO15	CD22	IgG2b	1:10	Dako

grounds of cost and staining quality. The immuno-staining was visualised with DAB (3,3'-diaminobenzidine)/hydrogen peroxide substrate solution. DAB was applied to each section at a concentration of 0.5mg/ml with 0.1 ml of 1% hydrogen peroxide for 4 minutes. After the visualisation step, the tissue sections were washed in water, counter-stained in haematoxylin (BDH Chemicals Ltd.) and 'blued up' in Scott's tap water. They were dehydrated in ascending grades of alcohol and mounted in xylene with Pertex mounting fluid (Cellpath Ltd.).

### **2.8.3 Indirect Peroxidase Method**

Several monoclonal antibodies cannot tolerate formalin fixation (Table 2.2). In these cases, the antibodies were used on acetone-fixed frozen tissue sections and the immuno-staining was carried out using the Indirect Peroxidase Method.

The tissue sections stored at -20°C were allowed to come up to room temperature. After washing in TBS, the sections were incubated in normal rabbit serum as before. The slides were washed between each stage with TBS as before. After incubation in primary antibody for 30 minutes, horse-radish peroxidase conjugated secondary antibody was then applied to each section for 30 minutes. Immuno-staining was visualised with DAB as before (2.8.2) and the sections were then counter-stained, dehydrated and mounted as before.

Immuno-staining resulted in a brown reaction product as seen in Figures 3.1, 4.7, 4.8, 5.3, 5.4, 6.1 & 6.2.

## 2.9 *In situ* Hybridisation

This method was used to detect the EBV encoded RNAs (EBER) in formalin-fixed paraffin sections using a fluorescein-conjugated EBER probe (Dakopatts).

To prevent RNase contamination, gloves were worn at all times throughout this technique. All glass-ware and instruments for use were washed over-night in Decon then heat-sterilised at 225°C for 4 hours. They were then stored covered in foil at a designated place exclusively for use with *in-situ* hybridisation. The use of DEPC (Diethylpyrocarbonate) treated water as a diluent for the various chemicals used in this technique also inhibited RNase.

The tissue sections were dewaxed in xylene and rehydrated through descending grades of alcohol. In order to expose the target RNA to the incoming probe, the sections were then digested with proteinase K (5µg/ml) for 1 hour in a moist chamber at 37°C. To stop any residual proteinase K activity, the sections were then post-fixed in pre-cooled 0.4% paraformaldehyde solution for 20 minutes at 4°C. Non-specific binding was blocked with pre-hybridisation buffer (Appendix A) for at least an hour in a moist chamber at 37°C. The hybridisation solution was prepared by adding the fluorescein conjugated EBER probe at a dilution of 1:20 to the pre-hybridisation buffer. 20µl of this solution was applied to each section, covered with gelbond (FMC Bio-products) and incubated over-night at 37°C.

The second day of the technique involved visualisation of the *in situ* hybridisation. Following hybridisation, non-specifically bound probe was washed out of the sections by a series of washes in decreasing concentrations of SSC at 37°C. The sections were then washed in TBS with

added BSA (1%)/2mM MgCl<sub>2</sub>/1% Triton-X 100 (BDH Chemicals Ltd.). The sections were also washed between each of the following stages in this TBS. Non-specific binding was blocked with normal swine serum before 30 minute incubations with the previously absorbed primary antibody (rabbit anti FITC) and the biotinylated swine anti rabbit secondary antibody. The Strept AB Complex/AP (Dakopatts Ltd.) was applied as 2 separate stages as this was found to be more sensitive. Streptavidin was applied first for 30 minutes before addition of biotinylated alkaline phosphatase for 30 minutes. The *in situ* was visualised using the alkaline phosphatase substrate BCIP/NBT (5-bromo-4-chloro-3-indoxyl phosphate/Nitro Blue Tetrazolium) (Appendix B). The visualisation step was carried out in the dark at 4°C. Staining was checked after 10 minutes and every 10 minutes thereafter - usually complete by 20-30 minutes. The BCIP/NBT mixture is soluble in alcohol and xylene, therefore Crystal Mount (Biogenesis Ltd.) was first applied to the sections before mounting in xylene/Pertex. No counter-stain was used. This resulted in a dark blue/purple reaction product as seen in Figures 5.1 & 5.2.

## **2.10      Enzyme Linked Immuno-Sorbent Assay** **(ELISA)**

This method was used to detect human immunoglobulin in the SCID mice sera.

SCID mice sera were diluted 1:100 and 1:1000 in carbonate/bicarbonate buffer pH9.6 and applied as the first layer (100µl/well) on a 96 micro-well ELISA plate (Nuncclon Ltd.). Normal human serum and normal mouse serum were also applied (both at 1:3000 dilution)

to serve as positive and negative controls respectively. This plate was incubated at 4<sup>0</sup>c over-night. The plates were washed x3 with washing buffer (PBS/0.1% BSA/0.05%Tween 20) prior to incubation with serial dilutions of horse-radish peroxidase conjugated goat anti-human polyvalent immunoglobulin or rabbit anti-mouse immunoglobulin ( to check that the SCID mouse sera did not contain any mouse immunoglobulin). These were diluted in PBS/1% BSA, and incubated for 2-3 hours at room temperature or over-night at 4<sup>0</sup>c. After washing x3 as before, the ELISA was visualised using the chromogen O-Phenylene Diamine (OPD) in citrate/phosphate buffer pH5.0 (0.2M Na<sub>2</sub>HPO<sub>4</sub> (M&B Lab Chemicals)/0.1M citric acid (BDH Chemicals Ltd.)). This was applied at a concentration of 0.4mg/ml with the addition of H<sub>2</sub>O<sub>2</sub> just before use. The colour was allowed to develop until the negative control wells were showing colour (usually 10 - 15 minutes).

Absorbance was measured using a Dynatech MR5000 plate reader. A positive result was recorded if the reading was higher than the negative control. Actual readings were recorded by subtraction of the negative reading. If a particular SCID serum was found to contain human immunoglobulin, then the specific immunoglobulin isotype was determined using peroxidase conjugated goat anti-human IgG, M, A, kappa and lambda. All of the positive SCID sera were run on the same plate in quadruplicate at a concentration of 1:1000. All of the secondary antibodies were applied at a concentration of 1:500. The 5 plates (i.e. IgM, G, A, kappa and lambda) were run on the same day and included positive (normal human serum) and negative (normal SCID serum, coating buffer only wells) controls.

Towards the end of the project, the SCID mice sera showing no trace of human immunoglobulin at the previous dilutions used were re-checked where possible using the lowest feasible dilution (1:2).



## **2.11      Flow Cytometry for DNA Ploidy**

This was used to measure the DNA ploidy of all original biopsies and SCID tumours. For the original Hodgkin's and Non-Hodgkin's lymphoma biopsies, parts of the lymph node considered to have the greatest number of RS/H cells or the most disease involvement were selected for use.

Nuclei suspensions were extracted from paraffin sections using the method of Hedley et al (1983). Briefly, 50µm paraffin sections were de-waxed in xylene, then rehydrated through descending grades of alcohol into distilled water. The sections or areas on slides were then digested with a 1ml solution of 0.5% pepsin in 0.9% NaCl (pH 1.5) for 1 hour at 37°C to obtain a nuclear suspension. The sections were vortexed every 10 minutes. After incubation, the sections were washed thoroughly in PBS by centrifugation then filtered through cotton wool and the nuclei were then stained with a 100µg/ml solution of propidium iodide/0.04% RNase for an hour at room temperature.

DNA ploidy was measured on a Coulter Epics CS flow cytometer. An argon laser tuned to 488nm was used with a 570 long pass filter. The PMT gains were kept the same for every sample analysed, the diploid peak occurred between channel number 43-48 for each sample. 10,000 nuclei were counted and the resulting histogram analysed using Linear S Phase Analysis on Cytologic Histogram Analysis for FCM on the Easy-2 program. The computer subtracted back-ground from the histogram before cell cycle analysis. The first peak was regarded as the diploid peak. Samples were considered to be aneuploid when in addition to the G0/G1 and G2/M peaks, one or more DNA peaks were detected. The DNA Index was calculated for all samples from the ratio of channel numbers for the aneuploid G0/G1 and

the diploid G0/G1 peak. Samples with DNA indices between 1.8 and 2.2 and in which the proportion of tetraploid cells exceeded 15% of the total cell count were regarded as tetraploid aneuploidy (Kallioneimi et al 1988). After analysis on the flow cytometer, every sample was checked under a fluorescence microscope to make sure no 'doublet nuclei' were present - 200 nuclei were counted, and in every case, under 2% of the population were found to be doublets.

The Molecular analyses of the original biopsies and the SCID tumours were carried out by Alice Gallagher in the Department of Veterinary Pathology in the University of Glasgow Veterinary School. Therefore, these protocols are given in Appendix C.

The following Chapters 3 - 8 are the results and discussion section of this thesis. Initially, the tumour yield from the HD and NHL biopsies is presented, then the characteristics of the SCID tumours are described and discussed. This includes morphology, phenotyping, EBV status, Ig gene rearrangement analysis, Ig expression and secretion and DNA ploidy.

## **Chapter Three**

### **Tumour Yield From SCID Mice**

#### **3.1 Tumour Yield**

A total of 42 fresh lymphoma biopsies were injected into SCID mice (each biopsy being injected into 4 or 5 mice). Of these, 17 were HD biopsies, the remaining 25 being NHL biopsies. 4 reactive lymph nodes were also injected.

5 cases of HD (29%) and 7 cases of NHL (28%) gave rise to human tumours in the SCID mice. None of the reactive lymph nodes gave rise to human tumours in the mice.

With the remaining 30 lymphoma biopsies, and including the cage-mates of the mice that developed tumours, a total of 184 mice were injected that did not develop human tumour.

Of these 184 mice, 28 (15%) were found dead in their cages, the time ranging from 3 weeks to 48 weeks after injection (mean was 25 weeks).

79 of these mice (43%) appeared to be sick (weight loss, hunched appearance, poor coat condition) but upon dissection, no evidence of tumour growth was found. Occasionally, these mice had symptoms such as slight spleen or liver enlargement but the vast majority were asymptomatic. The time between biopsy injection and death in these mice ranged from 7-80 weeks (mean was 36 weeks).

22 of these mice (12%) were found to have developed murine thymic lymphomas - these were morphologically different from the human tumours showing a more monomorphic cellular pattern and also failing to express the

human leucocyte common antigen (LCA) (Figure 3.1). DNA analysis was carried out on one of these murine thymic lymphomas, no human DNA was detected. These murine tumours developed in mice aged between 24 and 52 weeks (mean was 35 weeks).

2 different infections arose in the Animal House during the 3 year period. Together these were responsible for the mortality of 51 SCID mice (28%).

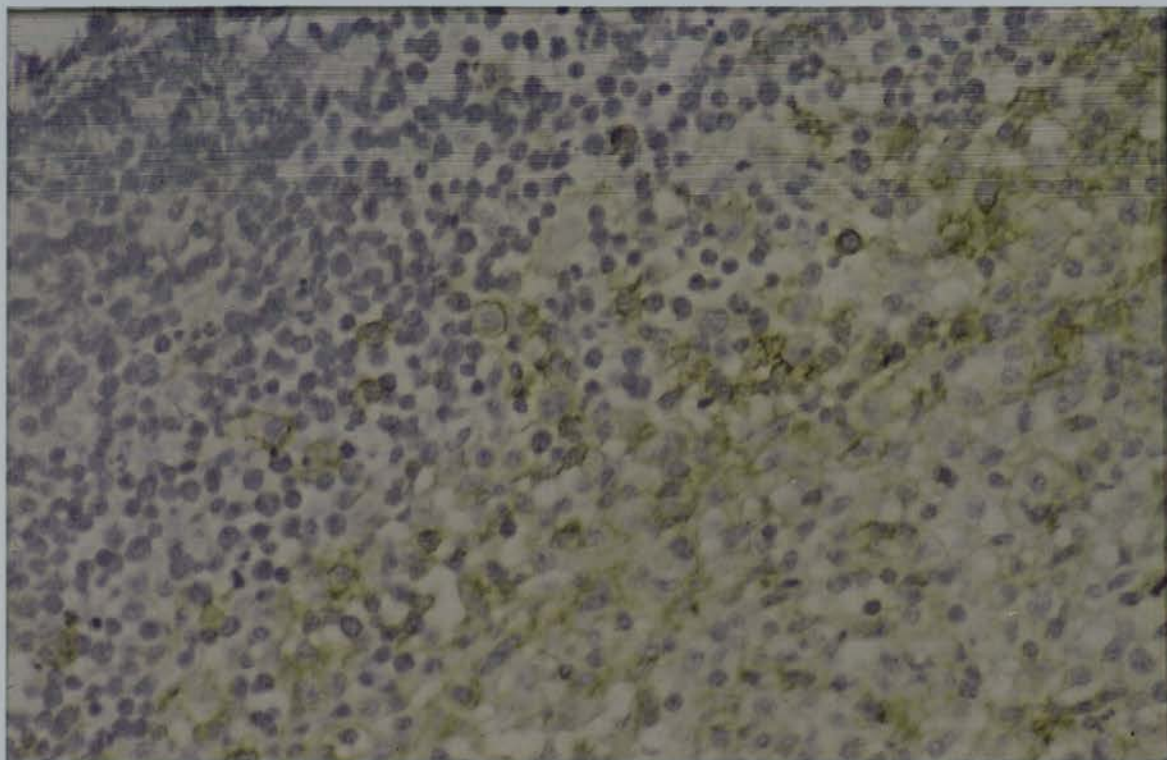
### **3.2 Post-Mortem Examination in Tumour Bearing SCID Mice**

In general, sick mice were underweight with a hunched appearance and a poor coat condition. Occasionally, laboured breathing was seen, and abdominal swelling was often present.

On dissection, the subcutaneous fatty tissue had a pinkish appearance and the superficial veins were often dilated with the appearance of inflammation. The lymph nodes were often visible. A main solid white tumour mass measuring around 2cm in diameter was found juxtaposed to the liver, stomach and spleen where the bulk of the pancreas would normally be sited. The tumour either invaded the liver tissue or simply surrounded it. The liver was often infarcted and enlarged as was the spleen. A macroscopic view of a typical SCID tumour is shown in Figure 3.2.

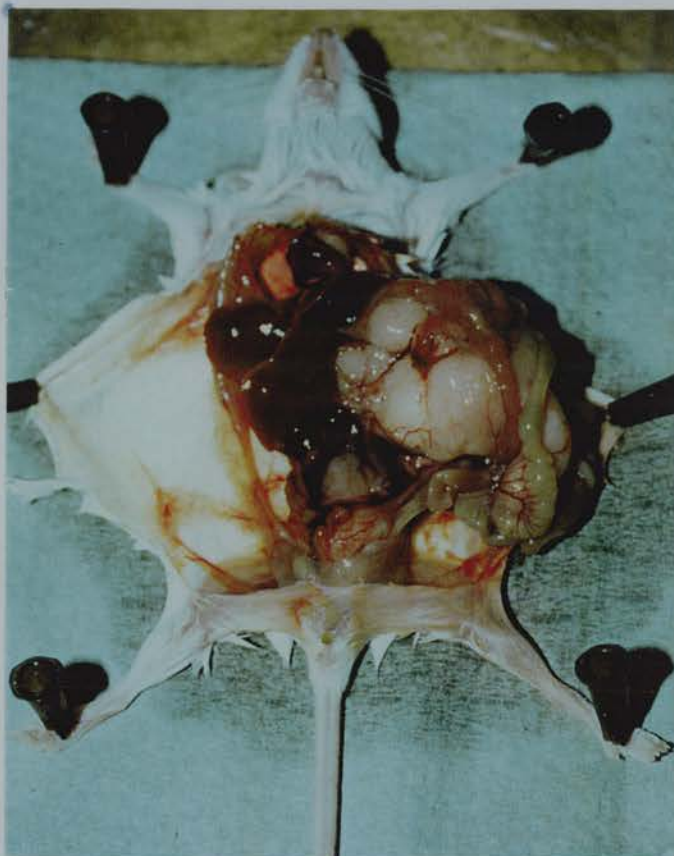
In some cases, all other abdominal organs appeared normal whilst in other cases, tumour nodules were found the length of the intestine (5mm diameter) and occasionally surrounding or invading the kidneys. Again in some cases the heart and lungs appeared normal with only the thymic remnant seen in SCID mice present, whereas in other cases thymic





**Figure 3.1** SCID Mouse Lung

This shows a section from a SCID mouse lung demonstrating human immunoblastic tumour (SCID tumour B2) expressing the human antigen CD43, growing along-side a murine thymic lymphoma showing no expression of the human CD43 antigen. A similar result was seen with the LCA. Mag x250



**Figure 3.2    Macroscopic View of a SCID Tumour**

This figure shows SCID tumour H1, but is typical of most of the SCID tumours, with a main tumour mass found in the liver and stomach region. In some cases, tumour nodules were also found the length of the intestine and surrounding the kidneys.

involvement was seen with either spread of the human tumour or a concurring murine thymic lymphoma.

### **3.3 Details of Human Tumours Arising in SCID mice**

Details of the original HD and NHL biopsies and the corresponding SCID tumours grown from these are shown in Tables 3.1 and 3.2.

The original HD and NHL biopsies are named Case A, Case B and so on upto Case L. Thus the HD biopsies are Cases A - E and the NHL biopsies are Cases F - L. The SCID tumours grown from transplantation of the original biopsies are given the same letter as the biopsy from which they were derived, plus a number. Even if there was only one SCID tumour grown from the injected biopsy, this is still given a number (i.e. 1) to distinguish it from the original biopsy. This nomenclature also allows distinction of different primary or passaged SCID tumours i.e. B1 and B2, K1, K2, K3 and K4. Collectively, the SCID tumours are referred to HD-derived or NHL-derived SCID tumours - this term is actually referring to the SCID tumour obtained from transplantation of the original HD or NHL biopsies, and does not infer that they have been proven to actually be derived from the malignant clone in the original biopsies.

SCID tumours were grown from 3 cases of LPHD (A, B, C), 1 case of NSHD (D) and 1 case of MCHD (E) (Table 3.1). Most of the HD cases only gave rise to 1 primary SCID tumour, the exception being Case C - LPHD which gave rise to 2 primary SCID tumours (C1 and C2). Of the HD-derived SCID tumours, only one case (B) passaged successfully - B1 is the primary tumour and the passaged tumour is denoted B2. There was no significant

**Table 3.1    Details of SCID Tumours Arising After Transplantation of Hodgkin's Disease**

ORIGINAL BIOPSY		SCID TUMOURS	
Case Number	Diagnosis	SCID Tumours	Latent Growth Period
A	LPHD	A1 - PRIMARY	11 wks
B	LPHD	B1 - PRIMARY B2 - PASSAGE	14 wks 12 wks
C	LPHD	C1 - PRIMARY C2 - PRIMARY	10 wks 10 wks
D	NSHD	D1 - PRIMARY	13 wks
E	MCHD	E1 - PRIMARY	12 wks

**Table 3.2   Details of SCID Tumours Arising After Transplantation of Non-Hodgkin's Lymphomas**

ORIGINAL BIOPSY		SCID TUMOURS	
Case Number	Diagnosis	SCID Tumours	Latent Growth Period
F	Centroblastic	F1 - F14  F1        PRIMARY F2-5     1st PASSAGE F6-9     2nd PASSAGE F10-13 3rd PASSAGE F14      4th PASSAGE	21 wks 15 wks 10 wks 9 wks 8 wks
G	Centroblastic	G1 - PRIMARY	29 wks
H	Centroblastic	H1 - PRIMARY	19 wks
I	Immunoblastic	I1 - PRIMARY	16 wks
J	Cb/Cc Foll	J1 - PRIMARY	20 wks
K	LCAL (B cell )	K1 - PRIMARY K2 - PRIMARY K3 - PRIMARY K4 - PRIMARY	9 wks 9 wks 9 wks 9 wks
L	LCAL (B cell)	L1 - PRIMARY L2 - PRIMARY L3 - PRIMARY L4 - PRIMARY L5 - PRIMARY	9 wks 11 wks 15 wks 15 wks 15 wks



difference in the number of weeks taken for each HD-derived SCID tumour to grow and this time did not appear to decrease on passage of B1.

SCID tumours were grown from 3 cases of centroblastic (F, G, H), 1 immunoblastic (I), 1 follicular (J) and 2 large cell anaplastic lymphomas, although the 2 LCAL cases (K and L) were actually sequential biopsies from the same patient (taken 4 weeks apart) (Table 3.2).

Cases F, G, H, I and J all only gave rise to one primary SCID tumour, whereas in Cases K and L, 100% SCID tumour development was achieved (4 and 5 primary SCID tumours respectively).

Case F was the only NHL case to produce SCID tumours that passaged successfully, and it did so very well - following passage of the only primary tumour (F1), 3 passages were carried out and gained 100% tumour take each time. SCID tumours F2-5 correspond to the first passage, F6-9 to the second and F10-13 to the third passage. After this, no more passages were carried out but a cell suspension from F13 was frozen down in liquid nitrogen. Later, this was thawed, reconstituted in RPMI/10% FCS and re-injected back into 4 SCID mice resulting in one further tumour (F14 - fourth passage).

There was no significant difference in the time taken for each NHL-derived SCID tumour to grow, being similar to that for the HD-derived tumours. The exception was tumour G1 which took slightly longer to develop (29 weeks).

A slight decrease in time taken for tumour growth was seen in the first passage of SCID tumour F, although this was not evident in the following passages.

Cell number injected into the mice did not appear to play any role in the development of the SCID tumours (range was  $0.7 - 7 \times 10^7$  cells).

### 3.4 Comment

Over a 3 year period, 42 lymphoma biopsies were available for injection into SCID mice. Although some biopsies received were insufficient to use in the project, this number was still lower than expected. Therefore the number of biopsies received was not favourable.

58% of the SCID mice used were found dead in their cages several weeks after injection or appeared sick but were found to be tumour-free. A large proportion of these mice would simply have died of old age. A small number of deaths were accidental due to a malfitted water bottle.

12% of the mice developed murine thymic lymphomas and this is in agreement with that reported for SCID mice (10-15%) (Custer et al 1985).

SCID tumours developed from 5/17 cases of HD and 7/25 cases of NHL. Each sub-type of Hodgkin's disease grew in the SCID mice (LDHD not being encountered). However, it is interesting that only 1 low grade NHL (Cb-Cc Foll - Case J) gave rise to a SCID tumour, the other 6 cases classified as high grade NHLs.

These lymphomas certainly appear to be more difficult to grow compared to other tumours such as melanoma, prostate and pancreatic carcinoma (Philips et al 1989) and to lymphomas induced by inoculation with EBV positive PBL (Cannon et al 1990, Nakamine et al 1991, Rowe et al 1991, Mosier et al 1992, Nadal et al 1992, Purtillo et al 1992, Veronese et al 1994)). However, in a similar study, Kapp et al (1993) only achieved growth of SCID tumours in 3/13 cases of HD injected - a slightly lower success rate than this project.

The time taken for these SCID tumours to grow matches that seen in a variety of tumours (the average latency period being 13 weeks) (Custer et

al 1985), and other HD-derived SCID tumours (11-20 weeks) (Kapp et al 1993).

Only 1 of the HD-derived SCID tumours and 1 of the NHL-derived SCID tumours passaged successfully. However, SCID tumour G1 was not passaged as it was too small and SCID tumour J1 was reported in my absence and therefore not passaged. 3 sets of 4 'passage' mice (from cases H, I and K) were killed by an Animal House infection. The HD-derived SCID tumour that did passage (B2) was too small for further passage. Therefore, it is possible that more of these SCID tumours would have passaged successfully.

## **Chapter Four**

### **Morphology and Phenotypic Analysis of Original Biopsies and SCID Tumours**

The morphology and phenotypic characteristics of the SCID tumours were investigated to determine how they compared to the original biopsy injected. Morphology was investigated using a standard Haematoxylin and Eosin stain. Phenotyping was carried out using a range of B cell, T cell , macrophage and activation markers (Tables 2.1 & 2.2).

#### **4.1 Morphology of the SCID Tumours**

In general, these SCID tumours showed numerous mitoses, some appearing abnormal, and apoptosis and necrotic areas were also found.

##### **SCID Tumour A1**

This tumour was derived from an LPHD (Case A). It was mainly comprised of immunoblast-type cells showing a prominent nucleolus. A few small lymphocytes were also present and cells with poly-lobated nuclei were also seen. A few large, multi-nucleated RS-like cells could be seen - in some cases these were surrounded by neutrophils. On the whole, this tumour appeared to be monomorphic showing only some plasmacytoid differentiation. This tumour invaded the liver however the gut and pancreas appeared normal, as were the kidneys, heart and lungs. The mesenteric lymph nodes were colonised by tumour.

### SCID Tumour B1

This tumour was also derived from an LPHD (Case B). It was also comprised of immunoblasts. There were many more small lymphocytes present in comparison with SCID tumour A1 and much plasmacytoid differentiation was seen. A few large cells with poly-lobated nuclei were seen as were large, multi-nucleated RS-like cells. Tumour infiltration was present in the mesenteric lymph nodes, liver and lung.

### SCID Tumour B2

This is the passaged SCID tumour from B1. Again, this was a mixture of immunoblasts some with poly-lobated nuclei, small lymphocytes, some plasmacytoid differentiation and a few multi-nucleated RS-like cells, some with surrounding macrophages. The tumour had invaded the mesenteric lymph nodes and liver but all other abdominal organs appeared normal. This mouse had also developed a murine thymic lymphoma and a mixture of human and murine lymphomas could be seen in the lungs (distinguished both morphologically and phenotypically) (Figure 3.1).

### SCID Tumours C1 and C2

These 2 primary SCID tumours were derived from a LPHD (Case C). Some areas of the C1 SCID tumour were monomorphic showing immunoblasts whereas other areas were made up of large pleomorphic cells with poly-lobated nuclei showing large nucleoli and more abundant cytoplasm, showing some features of large cell anaplastic lymphoma. The liver and mesenteric lymph nodes were invaded by tumour as were the lungs. All other organs appeared normal.

The other primary SCID tumour derived from Case C (designated C2) was not as pleomorphic as C1 showing more of an immunoblastic pattern



with some plasmacytoid differentiation present. The liver was invaded in this case with some tumour present in the lungs.

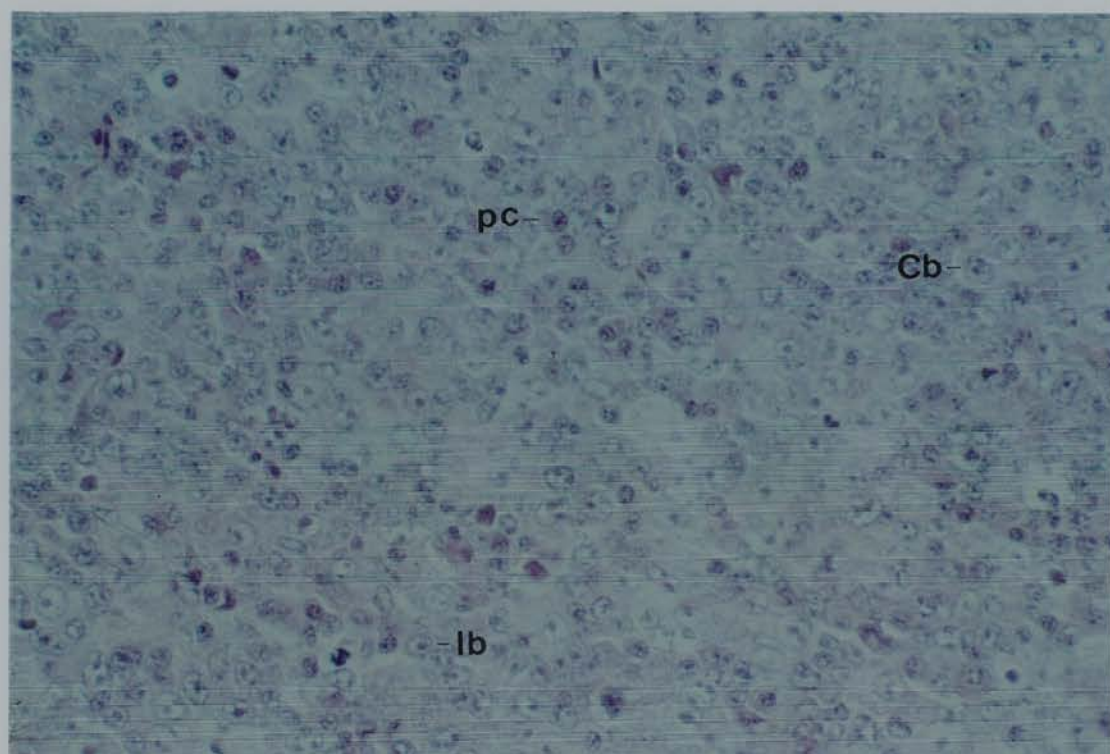
#### SCID Tumour D1

This SCID tumour was derived from a NSHD (Case D). Many large immunoblasts were present and a few centroblast-type cells. A few multi-nucleated cells were also present. There was a lot of plasmacytoid differentiation present and many classical plasma cells were seen. Some areas were made up of blast cells while other areas were comprised mainly of small lymphocytes. In this SCID tumour, there was a huge tumour mass invading the liver and a smaller mass near the stomach. The kidneys were invaded in this case.

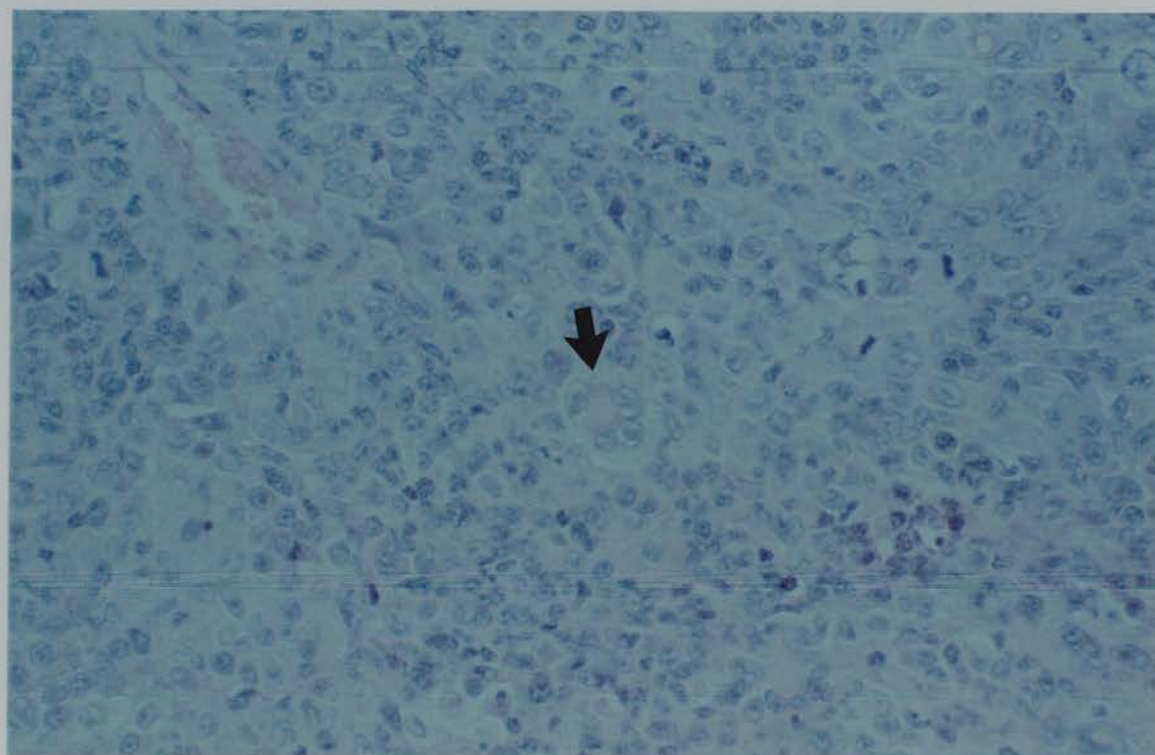
#### SCID Tumour E1

This SCID tumour was derived from a MCHD (Case E). It was comprised mostly of immunoblasts, some large showing poly-lobated nuclei. Some areas showed a mixture of blasts, small lymphocytes, plasma cells and a few multi-nucleated RS-like cells, whereas other areas showed undifferentiated blast cell proliferation. This SCID tumour remained localised to the site of injection (this was the only tumour to grow from a sc. injection) with no spread to any other organ.

The general morphological appearance of the HD-derived SCID tumours is depicted in Figure 4.1. The morphological appearance of the RS/H-like cells found in these SCID tumours is also shown in Figure 4.1. The LCAL-like features of SCID tumour C1 is shown in Figure 4.2.



General morphology showed immunoblasts, some centroblasts and plasmacytoid differentiation (SCID tumour D1). Mag x250



SCID tumour B1 showing an RS-like cell. Mag x250  
 arrow

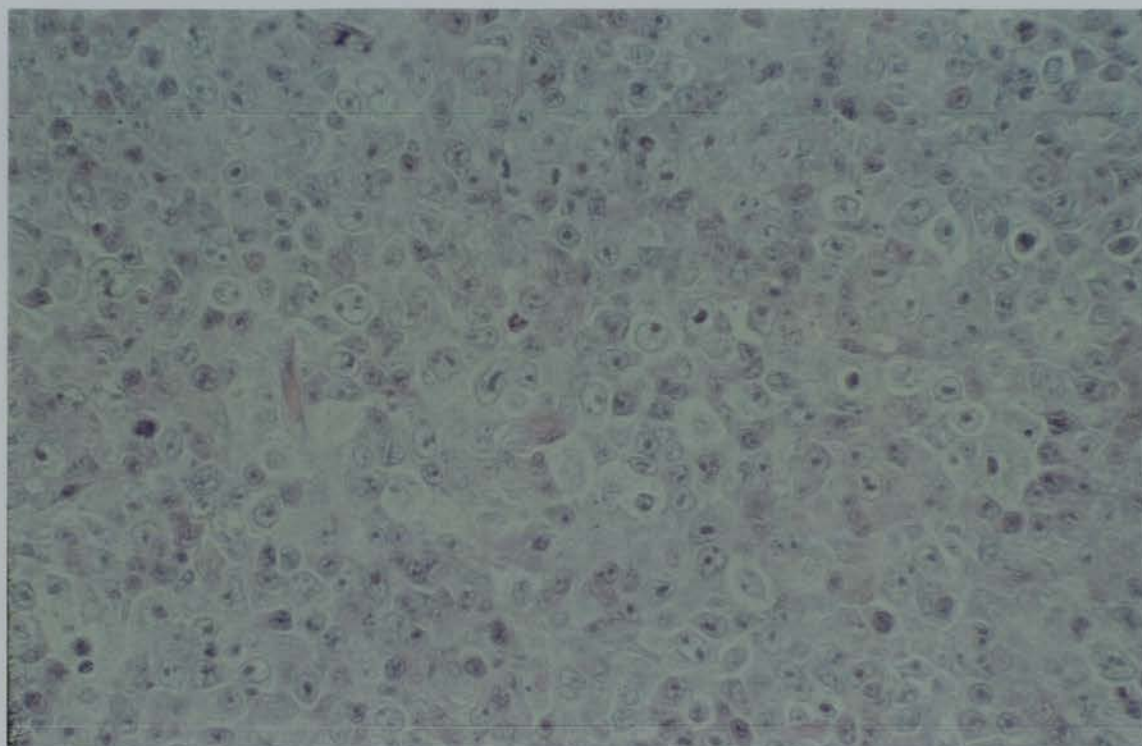
**Figure 4.1**    Morphology of the HD-Derived SCID Tumours

## **KEY FOR FIGURES 4.1 - 4.6**

Cb - centroblast

Ib - immunoblast

pc - plasmacytoid differentiation



**Figure 4.2** Morphology of SCID Tumour C1

This SCID tumour showed some LCAL-like features - the blasts cells are much larger, and have more abundant cytoplasm. Mag x250

### SCID Tumours F1 - F14

The original Case F biopsy was comprised of centroblasts, with some nuclear poly-lobation seen. This pattern was also seen in all of the SCID tumours, appearing morphologically identical to the original biopsy. There did not appear to be an increased nuclear poly-lobation throughout passage of the SCID tumours. In this set of SCID tumours, a main tumour mass was found in the pancreas area, in some cases the liver was invaded. In 5 cases, the tumour invaded the lungs. A distinctive pattern was seen in this set of SCID tumours which all had small discrete tumour nodules situated down the length of the intestine. In many cases, the tumour surrounded the kidneys (Figure 4.3).

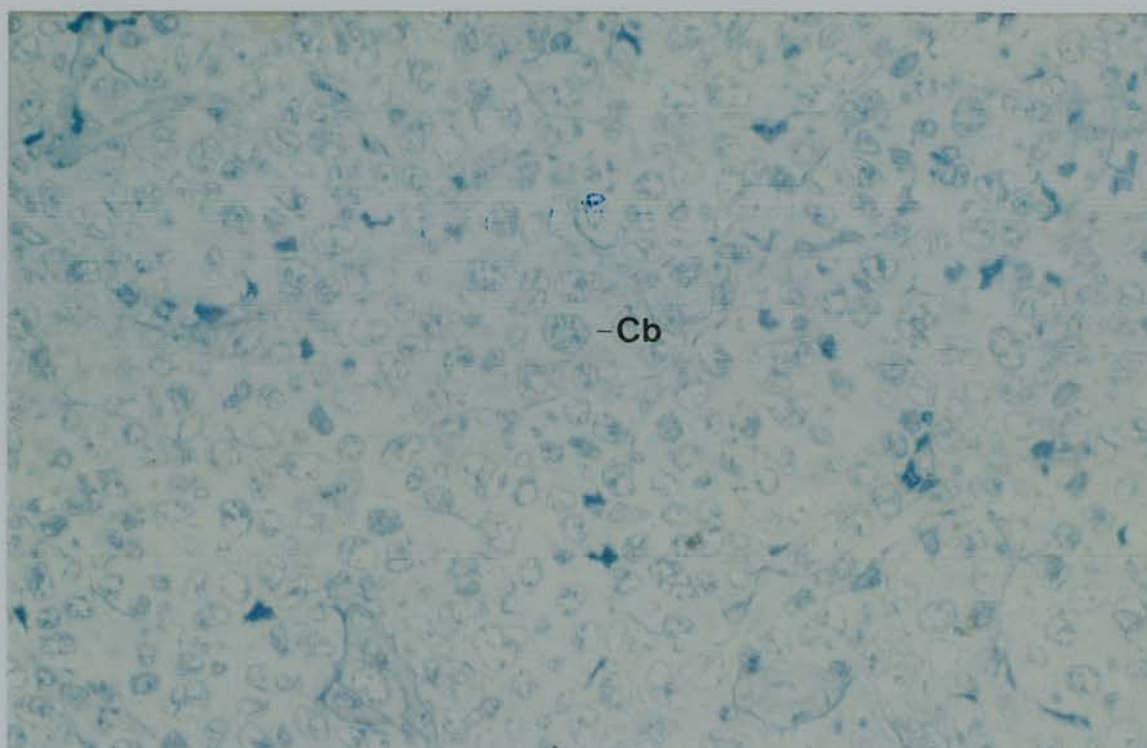
### SCID Tumour G1

This SCID tumour was derived from a centroblastic lymphoma (Case G). It was unusual in that it was only present associated with the male reproductive tract, in particular the epididymis. All other organs appeared normal. Morphologically, this SCID tumour was very similar to the original biopsy although it was slightly more pleomorphic as it contained both immunoblasts and centroblasts whereas the original biopsy was made up mostly of centroblasts (Figure 4.3 - see Figure 1.5 for morphology of the original biopsy Case G).

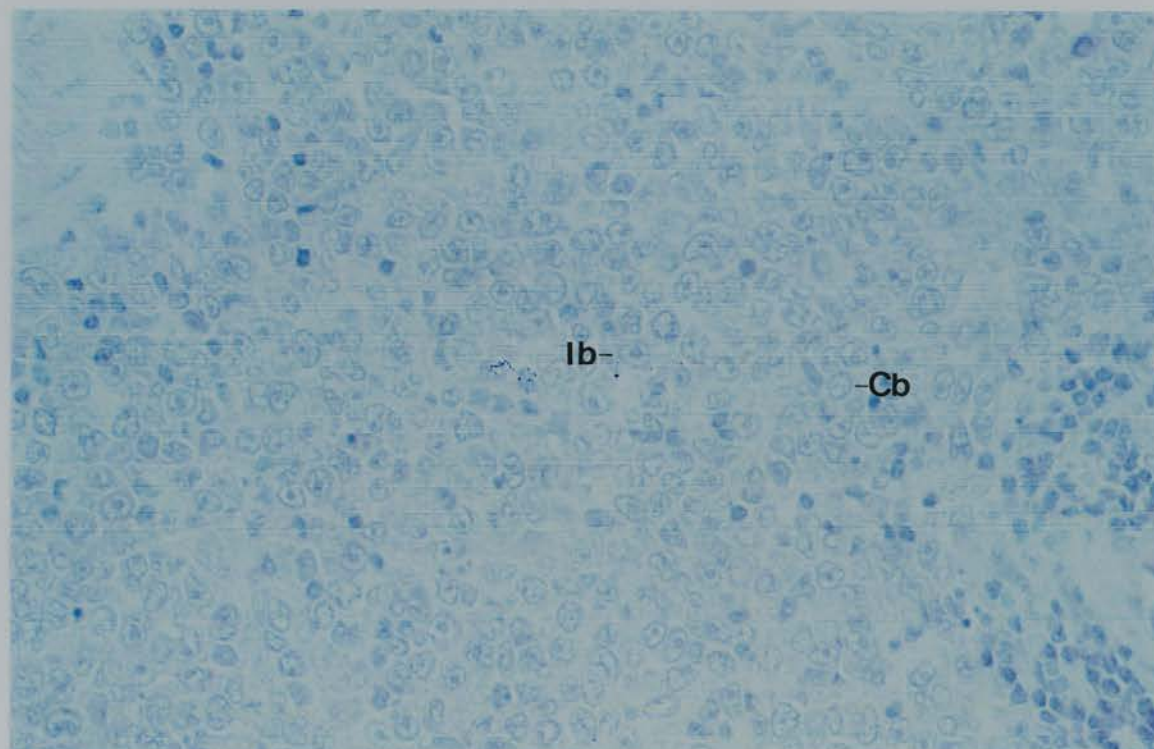
### SCID Tumour H1

Another centroblastic lymphoma (Case H) gave rise to this SCID tumour. The original biopsy was made up mostly of centroblasts with a few centrocytes. The SCID tumour looked very like the original although a mild degree of nuclear pleomorphism was seen with some poly-lobation.





SCID tumour F6, comprising centroblasts, some of which showed nuclear poly-lobation - this was morphologically identical to the original biopsy. Mag x250



SCID tumour G1. This was slightly more pleomorphic than the original biopsy as it contained both centroblasts and immunoblasts. Mag x250

#### Figure 4.3 SCID Tumour Morphology

However, it was comprised mostly of centroblasts with some immunoblasts present. This SCID tumour invaded the intestine only (Figure 4.4).

#### SCID Tumour I1

This was derived from an immunoblastic lymphoma (Case I). The original biopsy was comprised mainly of immunoblasts with very few centroblasts. The SCID tumour resembled this very closely although the immunoblasts here showed more abundant cytoplasm i.e. more plasmacytoid differentiation. This tumour invaded the mesentery and pancreas but all other organs were clear (Figure 4.4).

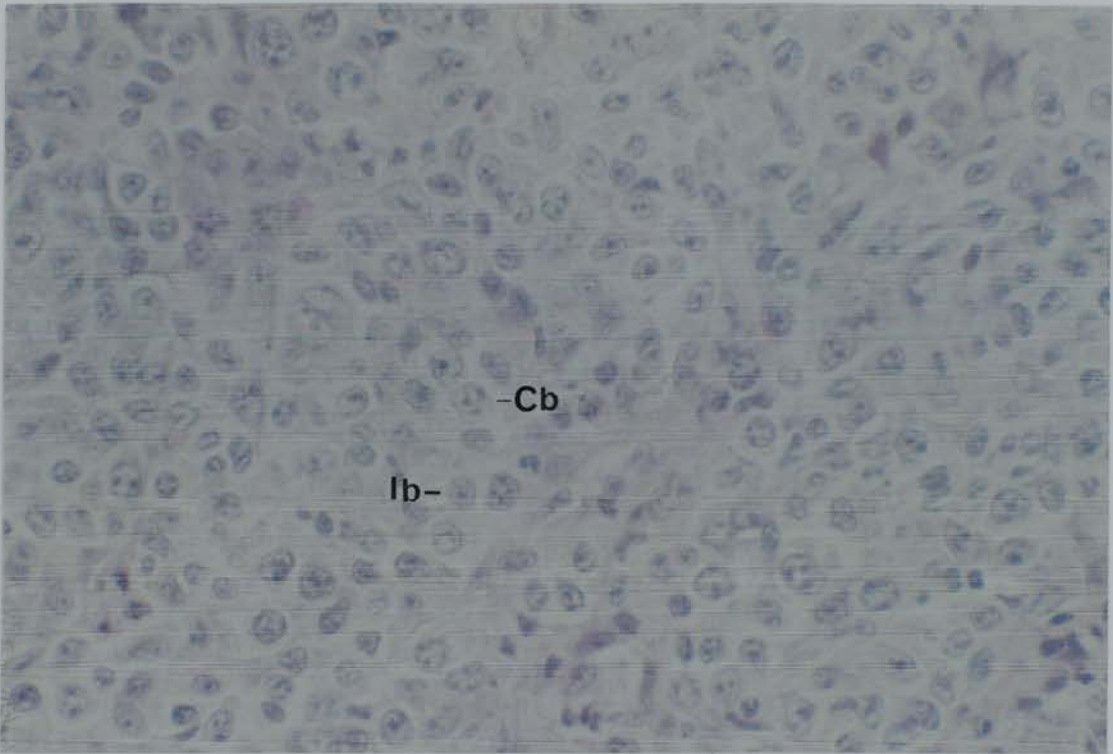
#### SCID Tumour J1

This SCID tumour was derived from a Cb-Cc Follicular lymphoma (Case J). The original biopsy showed a predominance of centrocytes over centroblasts. However, the SCID tumour did not appear to resemble the original biopsy, being comprised of a large immunoblastic proliferation. This SCID tumour showed extensive necrosis and did not exhibit a follicular pattern. This SCID tumour invaded the liver but all other organs were clear (Figure 4.5 - see Figure 1.5 for morphology of the original biopsy Case J).

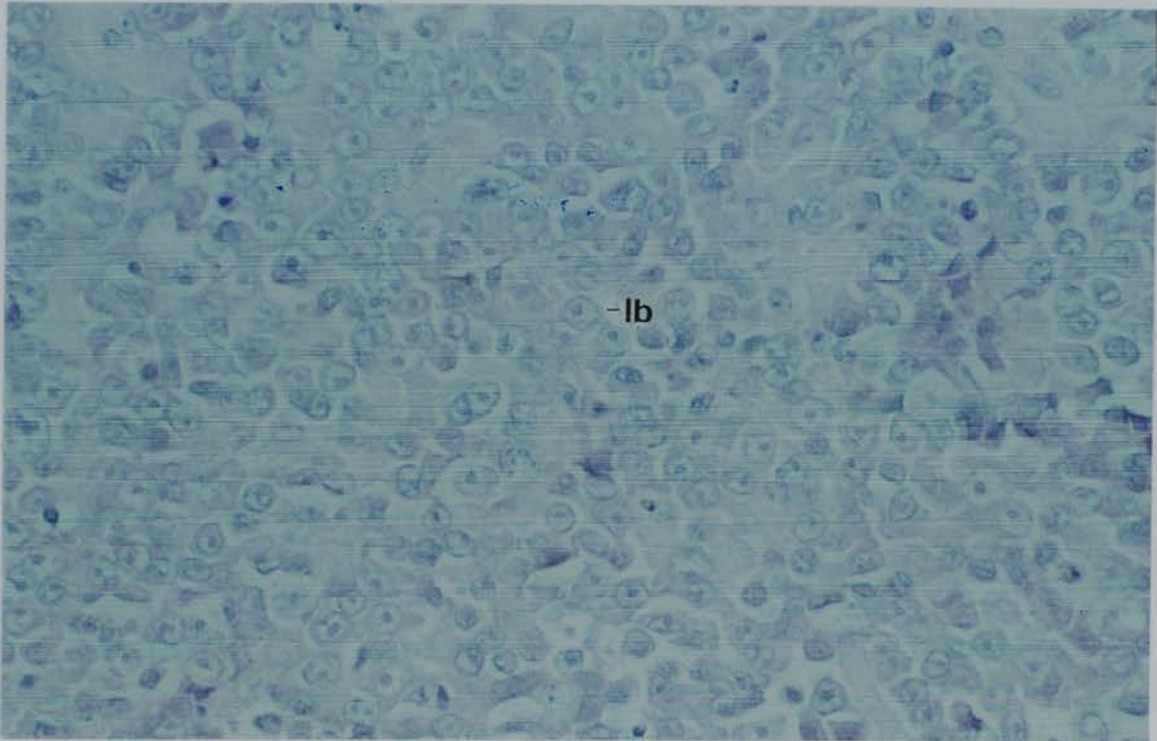
#### SCID Tumours K1 - K4

These 4 primary SCID tumours were derived from Case K - a B cell LCAL. The original biopsy showed a sinusoidal and paracortical infiltration of the malignant cells showing large, pleomorphic nuclei with one or more prominent nucleoli and abundant cytoplasm. Bi- and multi-nucleated forms were present.

SCID tumour K1 infiltrated the gut, liver, spleen, kidneys and mediastinal lymph node. In some areas, the tumour morphologically



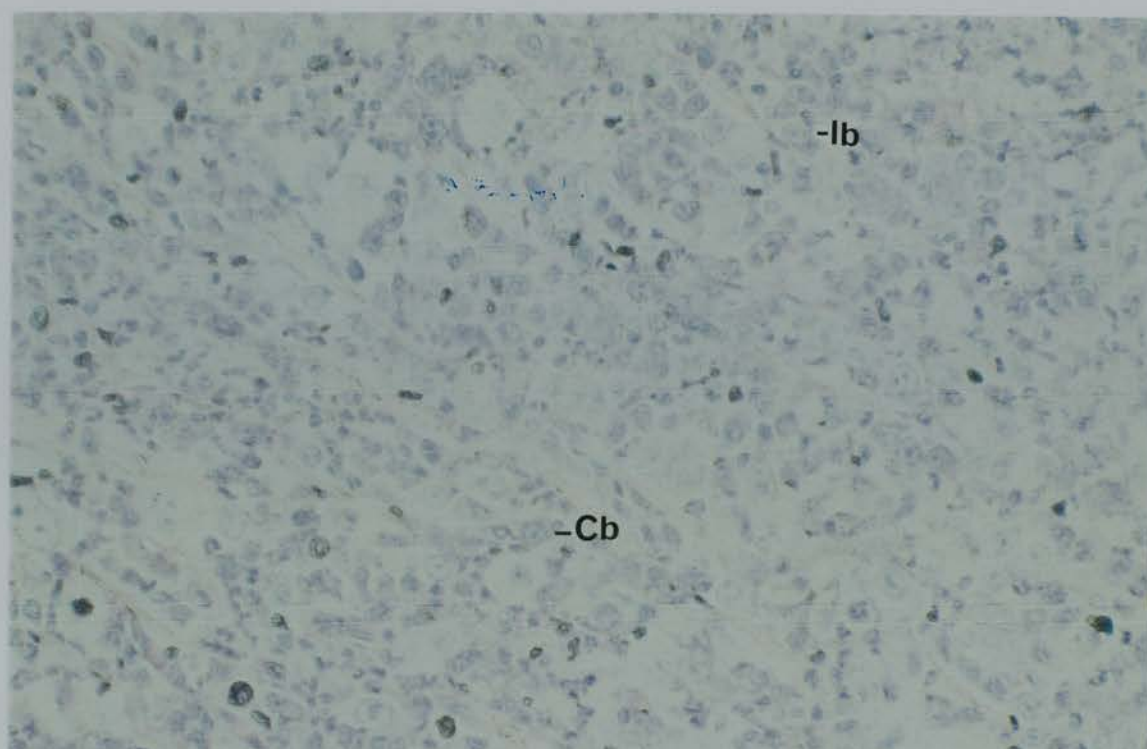
SCID tumour H1 being mostly comprised of centroblasts with a few immunoblasts. Mag x400



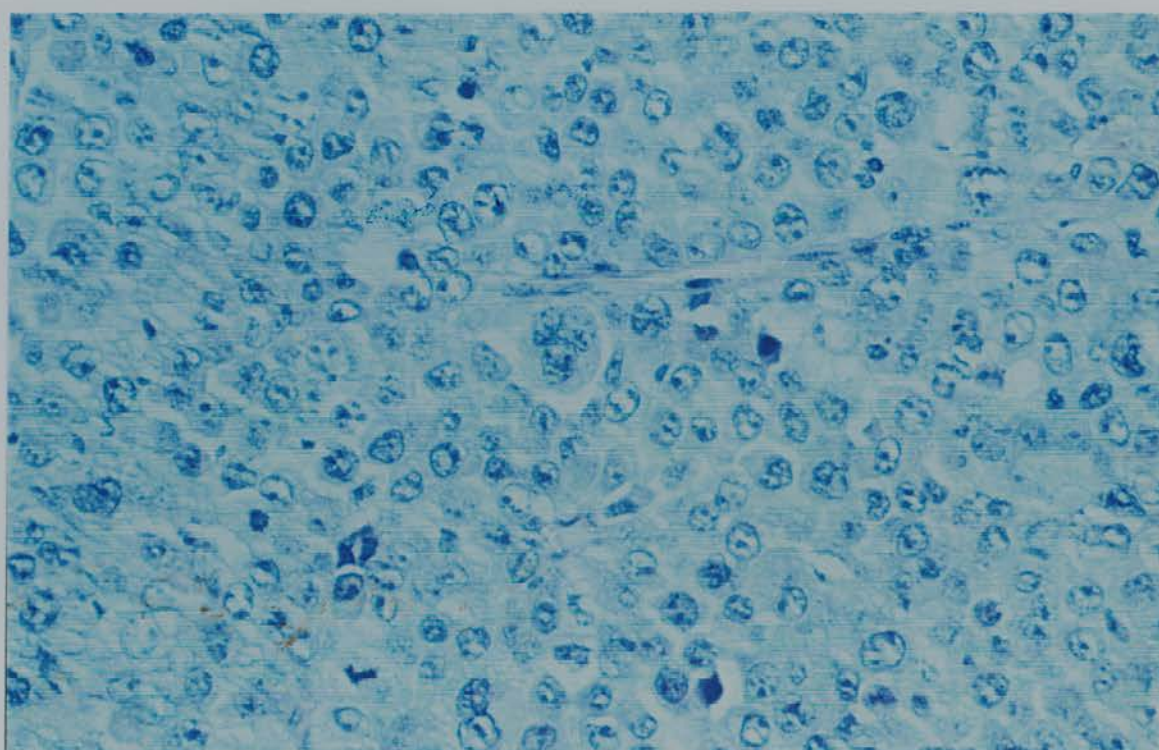
SCID tumour I1 being almost identical to the original biopsy. Mag x400

**Figure 4.4** SCID Tumour Morphology





SCID tumour J1 did not retain the morphology of the original biopsy, being mainly comprised of immunoblasts with a few centroblasts present. Mag x250



SCID tumour K2 showing an area which closely resembled the original biopsy. In other areas however, only a monomorphic immunoblastic proliferation was seen. Mag x250

**Figure 4.5** Morphology of SCID Tumours

resembled the original biopsy very closely and in other areas it appeared more polymorphous with large blasts, occasional multi-nucleated cells and some plasmacytoid differentiation. A small number of lymphocytes and plasma cells were also present.

SCID tumour K2 was not as invasive as K1, infiltrating only the liver.

Both SCID tumours K3 and K4 did not appear to be invading any organ but merely lying around them. They were all morphologically as K1 (Figure 4.5).

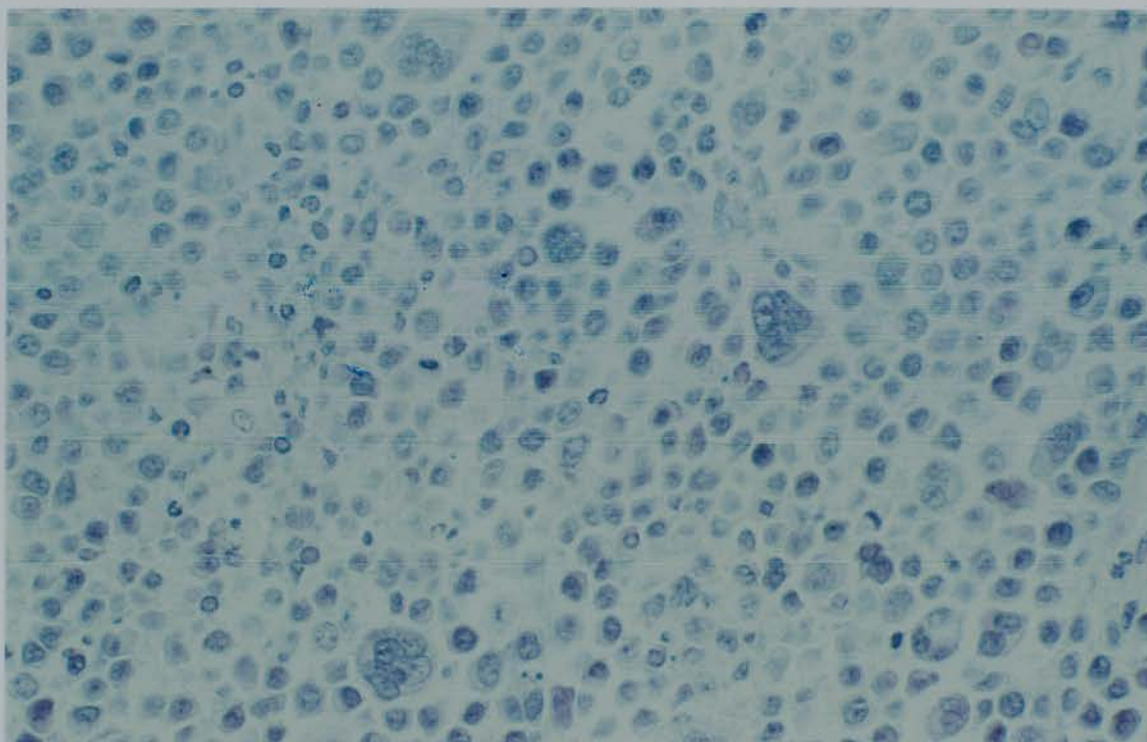
#### SCID Tumours L1 - L5

This case (Case L) was a sequential biopsy from Case K. This also showed a pleomorphic large cell infiltrate. The cells showed round and oval nuclei with prominent often multiple nucleoli. Bi-nucleate, multi-nucleate and polylobated nuclear forms were present.

SCID tumour L1 appeared to be histologically identical to the original biopsy perhaps showing more multi-nucleated forms. There also appeared to be small focal areas of lymphoproliferation with plasmacytoid differentiation and plasma cells present. This tumour spread into the liver, spleen, mesenteric lymph nodes and lungs.

SCID tumours L2, L3, L4 and L5 were much less invasive with the exception of L4 which infiltrated the liver. They all appeared histologically identical to the original biopsy, again with the exception of L4 in which focal areas of lymphoproliferation and plasmacytoid differentiation were additionally seen (Figure 4.6 - see Figure 1.6 for morphology of the original biopsy Case L).





SCID tumour L1 being morphologically identical to the original biopsy Case L, although containing more giant multi-nucleated tumour cells. Mag x250



In contrast, SCID tumour L4 contains areas of monomorphic immunoblastic proliferation shown here, although areas resembling the original biopsy were also present (see Figure 5.4). Mag x250

**Figure 4.6** Morphology of SCID Tumours

## **4.2 Phenotypic Characteristics of the Original Biopsies**

### **Hodgkin's Disease Biopsies**

The phenotypes of the RS/H cells in the HD biopsies are detailed in Tables **4.1**, **4.3** and **4.5**. Antigen expression, when detected, was seen in the majority, if not all, of the RS/H cells. As expected, Class II MHC (LN3) expression was ubiquitous. LCA expression was seen in the 3 LPHD cases (A, B & C) but absent in the other sub-types. The 3 LPHD cases (A, B & C) also expressed CD20, unusually for this sub-type of HD, Case B expressed CD15. Various B cell markers were expressed by all the cases of HD except Case E. No T cell or macrophage markers were expressed by any case. Both CD15 and CD30 were expressed by the NSHD (Case D) and the MCHD (Case E).

### **NHL Biopsies**

The phenotype of the NHL biopsies is detailed in Tables **4.2a**, **4.2b**, **4.4**, **4.6a** and **4.6b**. As expected, in the 3 centroblastic, the immunoblastic and the follicular lymphomas (Cases F, G, H, I and J), the tumour cells expressed the LCA and various B cell markers, but failed to express any T or macrophage markers, although residual T cell and macrophages were detected in the lymph node. Case H centroblastic lymphoma was unusual in that it was the only case expressing the CD43 antigen. The LCAL (Cases K and L) showed a very restricted antigen expression, the malignant cells stained positively only with MB2 and EMA. Case L also showed some staining for CD23. This LCAL was unusual in that it did not express the Ki-1 antigen (CD30).

### 4.3 Phenotypic Characteristics of the SCID Tumours

The phenotypic results are shown in Tables 4.1, 4.2a, 4.2b, 4.3, 4.4, 4.5, 4.6a and 4.6b. The key set out below applies to all these tables:-

The immuno-staining was assessed in a semi-quantitative manner as follows:-

+++	over 70% positive cells
++	40-70% positive cells
+	10-30% positive cells
+/-	1-5% positive cells
NEG	no staining seen

**w** weak staining. All other results not denoted showed good moderate/strong staining.

Frozen section morphology is of a lower standard than paraffin section morphology, and this caused a few problems in the assessment of the immuno-staining.

**(d)** Difficult to assess

**PQ** Poor Quality. No assessment could be made.

**ND** Not Distinguishable. Applies to the RS/H cells in the HD biopsies frozen sections.

**NM** No Material. If the SCID tumour was very small, material was taken in formalin for paraffin sections only.

**NS** Not Stained. Refers to the EMA column in Table 4.6b.

The immuno-staining results for the HD biopsies refer to the RS/H cells only, and are given as positive (**POS**) and negative (**NEG**) only.

Each original biopsy and its corresponding SCID tumour(s) are shown together on each table for ease of comparison.

## **B Cell Markers**

Included in this section is the LCA (CD45) expression.

### **HD-Derived SCID Tumours**

Table **4.1** shows the B cell phenotype of the HD-derived SCID tumours. All of the HD-derived SCID tumours showed strong expression of the leucocyte common antigen (CD45) (Figure **4.7**) and the B cell markers CD20 (Figure **4.7**), CD22, CD23 and CD40 (exception was SCID tumour A1). The CD19, MB2 and CD45RA antigens were strongly expressed in some cases but not others and the MB1 marker was expressed weakly or not at all.

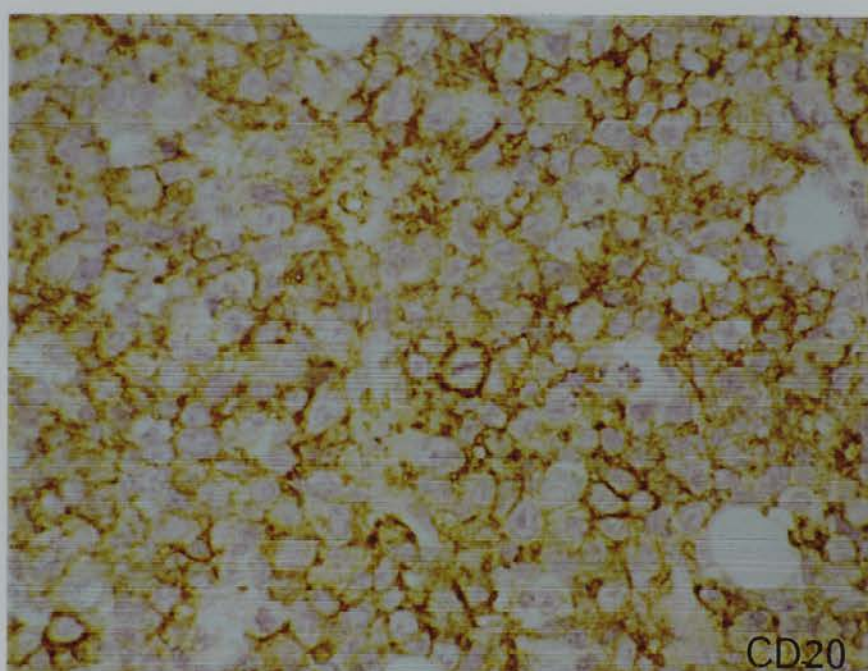
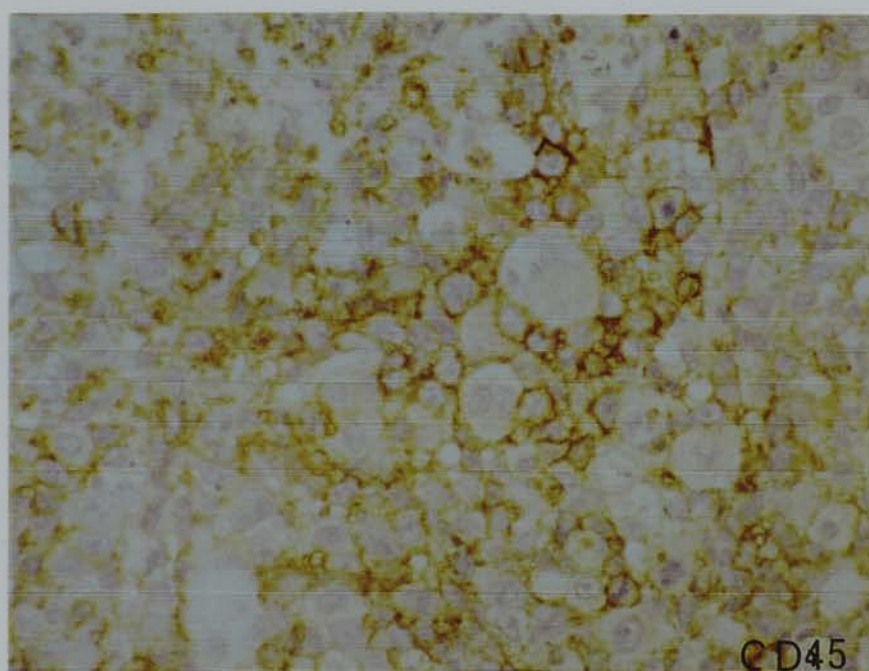
### **NHL-derived SCID Tumours**

The B cell phenotype of the SCID tumours derived from Case F centroblastic lymphoma (total = 14 including all the passages) are shown on Table **4.2a**. Each one (SCID F1 - F14) showed over 70% expression of LCA, as with CD20 and MB1 in 12/13 tumours - the exception being F1 having a lower percentage of CD20 and MB1 positive cells. In the case of MB2, CD45RA, CD22 and CD40, the majority of the Case F SCID tumours showed over 70% expression, the others showing over 50% expression. CD19 however was not as strongly expressed and CD23 was only weakly expressed in a minority of cells in one case (SCID tumour F4), the rest being CD23 negative.

The B cell phenotype of the SCID tumours from NHL cases G, H, I and J is shown in Table **4.2b**. They again all showed expression of LCA and L26 in the majority of the tumour cells.

SCID tumour G1 showed moderate expression of MB1 and MB2 and high CD40 expression. It also showed slight expression of the CD23





**Figure 4.7** Antigenic Expression of the SCID Tumours

SCID tumour A1 showing strong expression of both CD45 (LCA) (top) and CD20 (bottom).  
Mag x400



**Table 4.1 B Cell Antigen Expression in the Original Hodgkin's Disease Biopsies and SCID Tumours**

CASE (HD Biopsy/SCID tumour)	B CELL ANTIGEN EXPRESSION								
	LCA	CD20	CD19	CD22	MB1	MB2	CD45RA	CD23	CD40
Case A - LPHD SCID Tumour A1	POS +++	POS +++	ND +	ND ++	NEG NEG	NEG +++	NEG +	NEG +++	NEG +/-
Case B - LPHD SCID Tumour B1 SCID Tumour B2	POS +++ +++	POS ++ +++	ND +++ +++	ND +++ +++	POS w+ w+	POS +++ +++	NEG ++ +	NEG +++ +++	+/- +++ ++
Case C - LPHD SCID Tumour C1 SCID Tumour C2	POS ++ +++	POS +++ +++	ND +++ w+++	ND ++(d) ++(d)	w POS + +	NEG NEG NEG	w POS ++ ++	NEG +++ +++	NEG ++ ++
Case D - NSHD SCID Tumour D1	NEG +++	NEG ++	ND +/- (d)	ND +++	NEG +	POS +	NEG +	NEG +++	w+/- ++
Case E - MCHD SCID Tumour E1	NEG +++	NEG +++	ND ++	ND ++	NEG ++	NEG ++	NEG +++	NEG ++	NEG +++

**Table 4.2b B Cell Antigen Expression in NHL Biopsies and SCID Tumours**

**Table 4.2a B Cell Antigen Expression in NHL Biopsies and SCID Tumours**  
**(a) - Case F Centroblastic Lymphoma**

CASE F / SCID TUMOURS	B CELL ANTIGEN EXPRESSION									
	LCA	CD20	CD19	CD22	MB1	MB2	CD45RA	CD23	CD40	
Case F - NHL Cb	+++	++	NEG (d)	++	++	+++	+++	NEG	++	
SCID Tumour F1	+++	++	PQ	PQ	++	+++	+++	NEG	+++	
SCID Tumour F2	+++	+++	++	++	+++	w+	+++	NEG	+++	
SCID Tumour F3	+++	+++	w+	+++	+++	+++	+++	NEG	+++	
SCID Tumour F4	+++	+++	w+	PQ	+++	+++	+++	w+/-	+++	
SCID Tumour F5	+++	+++	w+	+++	+++	+++	+++	NEG	++	
SCID Tumour F6	+++	+++	++	+++	+++	+++	w+	NEG	+++	
SCID Tumour F7	+++	+++	++	++	+++	+++	+++	NEG	+++	
SCID Tumour F8	+++	+++	PQ	+++	+++	++	+++	NEG	+++	
SCID Tumour F9	+++	+++	++	+++	+++	+++	++	NEG	+++	
SCID Tumour F10	+++	+++	NM	NM	+++	+++	+++	NEG	+++	
SCID Tumour F11	+++	+++	NM	NM	+++	++	+++	NEG	+++	
SCID Tumour F12	+++	+++	++(d)	+++ (d)	+++	++	+++	NEG	+++	
SCID Tumour F13	+++	+++	++	+++	+++	++	+++	NEG	+++	
SCID Tumour F14	+++	+++	++	++	+++	++	++	NEG	+++	

Table 4.2b

**B Cell Antigen Expression in NHL Biopsies and SCID Tumours**  
**(b) - Remaining Cases**

CASE (NHL/SCID Tumours)	B CELL ANTIGEN EXPRESSION									
	LCA	CD20	CD19	CD22	MB1	MB2	CD45Ra	CD23	CD40	
Case G - NHL Cb SCID Tumour G1	+++	+++	NEG	+++	++	++	NEG	w++	NEG	
Case H - NHL Cb SCID Tumour H1	+++	+++	NM	NM	++	++	NEG	w++	+++	
Case I - NHL ImmB SCID Tumour I1	+++	+++	NEG	++	+++	+++	+	NEG	NEG	
Case J - NHL Cb/Cc Foll SCID Tumour J1	+++	+++	PQ	+++ (d)	+++	+++	+++	NEG	+++	
Case K - LCAL SCID Tumour K1	++	++	PQ	++	++	+++	+++	NEG	NEG	
Case L - LCAL SCID Tumour L1	+++	+++	NEG(d)	+++	+++	+++	+++	NEG	NEG	
SCID Tumour K2	++	++	w+++	+++	+++	NEG	w++	+++	++	
SCID Tumour K3	+++	+++	NEG(d)	+/-	NEG	++	NEG	+++	++	
SCID Tumour K4	+	++	NEG	NEG	NEG	w+++	NEG	NEG	NEG	
Case L - LCAL SCID Tumour L1	NEG	NEG	NEG	NEG	+	++	NEG	++	++	
SCID Tumour L2	+/-	+++	PQ	PQ	+	+++	+	+++	+	
SCID Tumour L3	NEG	+++	NM	NM	NEG	+++	++	+++	+/-	
SCID Tumour L4	+++	+++	NM	NM	NEG	w++	+/-	+++	NEG	
SCID Tumour L5	+	++	NM	NM	NEG	w+++	+	+++	NEG	
Case L - LCAL SCID Tumour L1	NEG	NEG	NEG	NEG	NEG	w+++	NEG	+	NEG	
SCID Tumour L2	+/-	+/-	PQ	PQ	NEG	+++	NEG	+++	NEG	
SCID Tumour L3	NEG	NEG	NEG	NEG	NEG	+++	NEG	+++	NEG	
SCID Tumour L4	NEG	NEG	NEG	PQ	NEG	+++	NEG	+++	NEG	
SCID Tumour L5	+/-	+/-	NEG	NEG	NEG	+++	NEG	+++	NEG	

antigen, retaining the phenotype of the original biopsy. No material was available for frozen section immuno-phenotyping.

SCID tumour H1 showed expression of all the B cell antigens except CD23 as did SCID tumour I1 although it showed no CD40 expression.

SCID tumour J1 showed moderate expression of the CD40 and MB2 antigens although high expression of the CD23 antigen was seen.

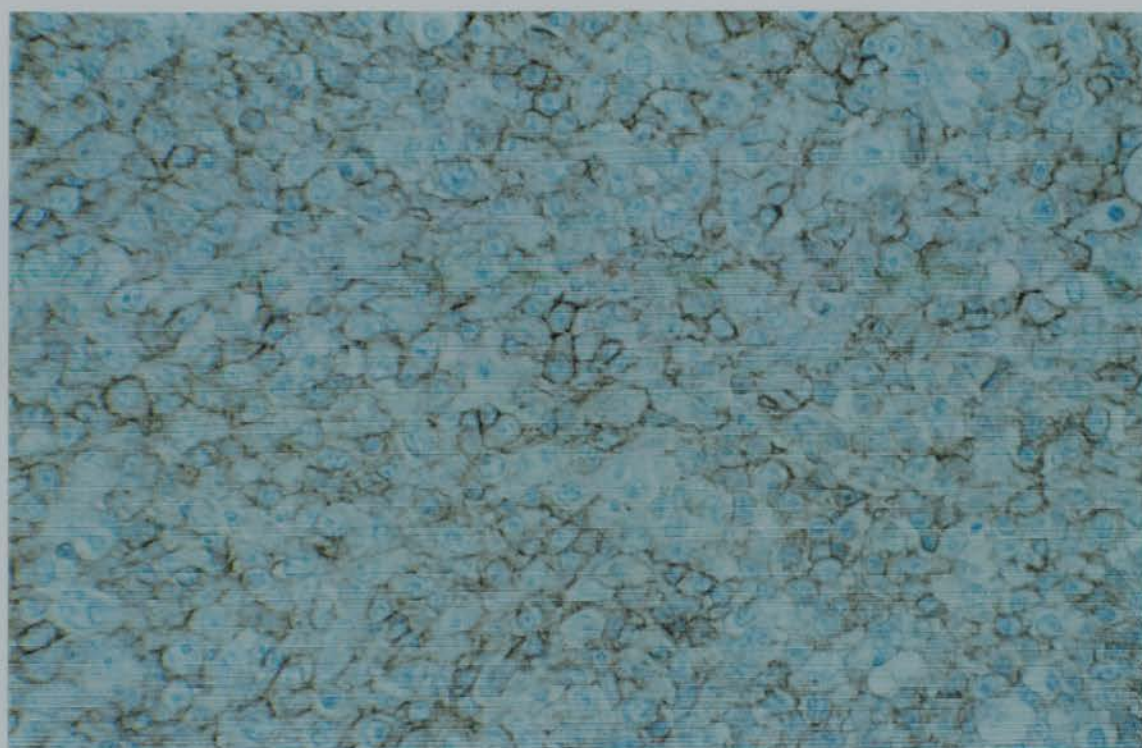
The 2 LCAL biopsies (Cases K and L) produced 2 phenotypically very different sets of SCID tumours. The first set (derived from Case K) showed LCA, CD20, MB2 and CD23 expression, less so CD45RA and MB1. In contrast, the SCID tumours derived from Case L showed a very limited phenotypic pattern - only showing expression of MB2 and CD23. The exception was SCID tumour L1 and L4 which only showed LCA and L26 expression in a minority of the SCID tumour cells, these cells being of the immunoblastic morphology described as present in small focal areas.

The LCAL biopsies and their SCID tumours were also stained for the epithelial membrane antigen (EMA) as this is commonly found in these lymphomas (Chan et al 1989). Both original biopsies were positively stained, however only 2 of the SCID tumours from case K stained with EMA and only in a minority of cells whereas all the SCID tumours from Case L were strongly positive with this marker.

### **T Cell Markers**

The SCID tumours were stained with the T cell markers CD3, CD4, CD8, CD43 and CD45Ro. None of the SCID tumours were positive for any of the T cell markers except the CD43 antigen (Figure 4.8). All of the HD-derived SCID tumours stained positively for this antigen. The NHL-derived SCID tumours from Cases H, J and K also expressed this antigen to varying degrees (Tables 4.3 and 4.4).





**Figure 4.8** CD43 Expression

SCID Tumour C2 showing high expression of the CD43 antigen.



**Table 4.3    T Cell Antigen Expression in Hodgkin's Disease Biopsies and SCID Tumours**

<b>CASE (HD Biopsy/SCID Tumour)</b>	<b>T CELL ANTIGEN EXPRESSION</b>					
	<b>pCD3</b>	<b>CD3</b>	<b>CD4</b>	<b>CD8</b>	<b>CD43</b>	<b>CD45Ro</b>
<b>Case A - LPHD SCID Tumour A1</b>	<b>NEG NEG</b>	<b>ND NEG</b>	<b>ND NEG</b>	<b>ND NEG</b>	<b>NEG +++</b>	<b>NEG NEG</b>
<b>Case B - LPHD SCID Tumour B1 SCID Tumour B2</b>	<b>NEG NEG NEG</b>	<b>ND NEG NEG</b>	<b>ND NEG NEG</b>	<b>ND NEG NEG</b>	<b>NEG ++ +++</b>	<b>NEG NEG NEG</b>
<b>Case C - LPHD SCID Tumour C1 SCID Tumour C2</b>	<b>NEG NEG NEG</b>	<b>ND NEG NEG</b>	<b>ND PQ NEG</b>	<b>ND PQ NEG</b>	<b>NEG +++ +++</b>	<b>NEG NEG NEG</b>
<b>Case D - NSHD SCID Tumour D1</b>	<b>NEG NEG</b>	<b>ND NEG</b>	<b>ND NEG</b>	<b>ND NEG</b>	<b>NEG +++</b>	<b>NEG NEG</b>
<b>Case E - MCHD SCID Tumour E1</b>	<b>NEG NEG</b>	<b>ND NEG</b>	<b>ND NEG</b>	<b>ND NEG</b>	<b>NEG +++</b>	<b>NEG NEG</b>

**Table 4.4    T Cell Antigen Expression in Non-Hodgkin's  
Lymphoma Biopsies and SCID Tumours**

CASE (NHL Biopsy/SCID Tumour)	T CELL ANTIGEN EXPRESSION					
	pCD3	CD3	CD4	CD8	CD43	CD45Ro
Case F - NHL Cb SCID Tumours F1-F14	NEG NEG	NEG NEG	NEG NEG	NEG NEG	NEG NEG	NEG NEG
Case G - NHL Cb SCID Tumour G1	NEG NEG	NEG NM	NEG NM	NEG NM	NEG NEG	NEG NEG
Case H - NHL Cb SCID Tumour H1	NEG NEG	NEG NEG	NEG NEG	NEG NEG	++ +++	NEG NEG
Case I - NHL ImmB SCID Tumour I1	NEG NEG	NEG NEG	NEG NEG	NEG NEG	NEG NEG	NEG NEG
Case J - NHL Cb/Cc Foll SCID Tumour J1	NEG NEG	NEG NEG	NEG NEG	NEG NEG	NEG ++	NEG NEG
Case K - LCAL SCID Tumour K1 SCID Tumour K2 SCID Tumour K3 SCID Tumour K4	NEG NEG NEG NEG NEG	NEG PQ NM NM NM	NEG PQ NM NM NM	NEG PQ NM NM NM	NEG ++ +++ +++ +/-	NEG NEG NEG NEG NEG
Case L - LCAL SCID Tumour L1 SCID Tumour L2 SCID Tumour L3 SCID Tumour L4 SCID Tumour L5	NEG NEG NEG NEG NEG NEG	NEG NEG NEG NEG NEG NEG	NEG NEG NEG NEG NEG NEG	NEG NEG NEG NEG NEG NEG	NEG NEG NEG NEG NEG NEG	NEG NEG NEG NEG NEG NEG

**Table 4.5    Antigen Expression in Hodgkin's Disease Biopsies and SCID Tumours - Macrophage & Other Markers**

CASE (HD Biopsy/ SCID Tumour)	ANTIGEN EXPRESSION							
	CD15	CD30	LN3	CD25	CD18	CD11b	CD14	CD68
Case A - LPHD SCID Tumour A1	NEG NEG	POS ++	POS +++	ND +/-	ND +++	ND NEG	ND NEG	NEG NEG
Case B - LPHD SCID Tumour B1 SCID Tumour B2	POS NEG NEG	NEG w+ w+	POS +++ ++	ND +/- +/-	ND + ++	ND NEG NEG	ND NEG NEG	NEG NEG NEG
Case C - LPHD SCID Tumour C1 SCID Tumour C2	NEG NEG NEG	NEG +++ +++	POS ++ +++	ND PQ NEG	ND +++ +++	ND NEG NEG	ND NEG PQ	NEG +/- NEG
Case D - NSHD SCID Tumour D1	POS NEG	POS +++	POS +++	ND +/-	ND ++(d)	ND +(d)	ND NEG	NEG NEG
Case E - MCHD SCID Tumour E1	POS ++	POS w+	POS +++	ND +/-	ND ++	ND NEG	ND NEG	NEG NEG

Table 4.6a

**Antigen Expression in Case F Centroblastic Lymphoma and SCID Tumours - Macrophage & Other Markers**

CASE F/SCID TUMOURS	ANTIGEN EXPRESSION							
	CD15	CD30	LN3	CD25	CD18	CD11b	CD14	CD68
Case F - NHL Cb	NEG	NEG	++	PQ	++	NEG	NEG	NEG
SCID Tumour F1	NEG	NEG	++	+	+	NEG	NEG	NEG
SCID Tumour F2	NEG	NEG	+++	+	NEG	NEG	NEG	NEG
SCID Tumour F3	NEG	NEG	+++	+	+	NEG	NEG	NEG
SCID Tumour F4	NEG	NEG	+++	NEG	+(d)	NEG	NEG	NEG
SCID Tumour F5	NEG	NEG	+++	NEG	+(d)	PQ	NEG	NEG
SCID Tumour F6	NEG	NEG	+++	+	++(d)	NEG	NEG	NEG
SCID Tumour F7	NEG	NEG	+++	NEG	++	+(d)	NEG	NEG
SCID Tumour F8	NEG	NEG	++	+(d)	++	NEG	NEG	NEG
SCID Tumour F9	NEG	NEG	+++	+	++	+	NEG	NEG
SCID Tumour F10	NEG	NEG	+++	NM	NM	NM	NM	NEG
SCID Tumour F11	NEG	NEG	+++	NM	NM	NM	NM	NEG
SCID Tumour F12	NEG	NEG	+++	+(d)	++	+	NEG	NEG
SCID Tumour F13	NEG	NEG	+++	NEG	++	+	NEG	NEG
SCID Tumour F14	NEG	NEG	+++	NEG	++	+	NEG	NEG

Table 4.6b

**Macrophage and Other Markers in NHL Biopsies and SCID Tumours**  
**(b) - Remaining NHL Cases**

CASE	ANTIGEN EXPRESSION									
(NHL/SCID TUMOURS)	CD15	CD30	LN3	CD25	CD18	CD11b	CD14	CD68	EMA	
Case G - NHL Cb SCID Tumour G1	NEG	NEG	+++	NEG	PQ	PQ	NEG	NEG	NS	
	NEG	+	+++	NM	NM	NM	NM	NEG	NS	
Case H - NHL Cb SCID Tumour H1	NEG	NEG	++	PQ	PQ	PQ	NEG	NEG	NS	
	NEG	+/-	+++	PQ	PQ	NEG	NEG	+/-	NS	
Case I - NHL Immb SCID Tumour I1	NEG	NEG	++	NEG	NEG	PQ	NEG	NEG	NS	
	NEG	w+	+++	NEG	NEG	PQ	NEG	NEG	NS	
Case J - NHL Cb/Cc Foll SCID Tumour J1	NEG	+/-	+++	NEG	+++ (d)	NEG	NEG	NEG	NS	
	NEG	++	++	NEG	NEG	NEG	NEG	+/-	NS	
Case K - LCAL SCID Tumour K1	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	+++	
	NEG	++	++	PQ	PQ	PQ	PQ	NEG	NEG	
SCID Tumour K2	NEG	++	++	NM	NM	NM	NM	NEG	+/-	
SCID Tumour K3	NEG	++	++	NM	NM	NM	NM	NEG	NEG	
SCID Tumour K4	NEG	++	++	NM	NM	NM	NM	NEG	+/-	
Case L - LCAL SCID Tumour L1	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	+++	
	NEG	+++	NEG	NEG	NEG	NEG	NEG	NEG	+++	
SCID Tumour L2	NEG	+++	NEG	NEG	NEG	NEG	NEG	NEG	++	
SCID Tumour L3	NEG	+++	NEG	NEG	NEG	NEG	NEG	NEG	+++	
SCID Tumour L4	NEG	+++	NEG	PQ	NEG	PQ	NEG	NEG	+++	
SCID Tumour L5	NEG	+++	NEG	NEG	NEG	NEG	PQ	NEG	+++	



### **Monocyte/Macrophage Markers**

The SCID tumours were stained with the CD14 monocyte marker and the CD68 macrophage marker. Although none of the SCID tumours expressed the CD14 antigen, in a few cases (SCID tumours C1, H1 and J1) a few CD68-expressing macrophages were seen. The CD11b antigen was only expressed on a minority of cells in SCID tumours D1 and a few of the passaged Case F SCID tumours (Tables 4.5, 4.6a and 4.6b).

### **Other Markers**

The SCID tumours were stained against the 2 antigens associated with HD - CD15 and CD30. All of the NHL-derived SCID tumours were CD15 negative, however one HD-derived SCID tumour (E1) expressed CD15 (retaining the phenotype of the original biopsy). All of the HD-derived SCID tumours expressed CD30 to varying degrees. CD30 was not expressed in any Case F SCID tumour but was expressed in varying degrees in the remaining NHL-derived SCID tumours including Case L LCAL.

All of the SCID tumours showed good expression of Class II MHC and most with the CD18 LFA-1 antigen (except the SCID tumours L1 - L5), however IL-2R (CD25) expression was very limited (Tables 4.5, 4.6a and 4.6b).

## **4.4 Comment**

### **HD-Derived SCID Tumours**

In general, transplantation of HD into SCID mice produced high grade, B cell immunoblastic lymphomas with a mixture of smaller lymphocytes and areas of plasmacytoid differentiation. None of the HD-derived SCID tumours

showed the morphological picture seen in HD, but SCID tumours C1 and C2 showed some features of LCAL and areas of increased cellular polymorphism could be seen in most cases. Occasional bi-nucleate or multi-nucleated RS-like cells were also found in each of the HD-derived SCID tumours.

As expected, the 3 LPHD expressed CD20 and various other B cell antigens (Norton et al 1987, Hall et al 1988b, Nicholas et al 1990, Poppema 1992), however the NSHD (Case D) only showed MB2 and weak CD40 expression and the MCHD (Case E) did not express any B cell markers. The HD-derived SCID tumours did not follow the phenotypic pattern of the original biopsies, instead exhibiting an activated B cell phenotype. Although the RS/H cells in all the HD cases (except Case E MCHD) expressed various B cell markers, the same pattern and intensity of expression was not matched in the SCID tumours (comparison of CD19 and CD22 expression between the original biopsies and SCID tumours cannot be considered here as RS/H cell staining could not be determined because of poor frozen section morphology). Instead, the HD-derived SCID tumours displayed a mature, activated B cell phenotype regardless of injected HD biopsy RS/H cell phenotype. No B cell antigen expression was lost in the SCID tumours - a notable pattern was the expression of the CD23 antigen, a B cell activation antigen associated with EBV (Wang et al 1990), which was not expressed by the RS/H cells in any of the HD biopsies but highly expressed in all of the HD-derived SCID tumours.

None of the SCID tumours expressed the HD-associated antigen CD15 despite 3 original cases expressing this antigen. The exception was Case E MCHD in which the SCID tumour E1 retained the original phenotype. All of the HD-derived SCID tumours expressed the CD30 antigen to one degree or another - however this was seen even when the original HD cases

were negative (Cases B and C) and may well simply reflect the activation phenotype of the SCID tumour blasts. However, CD30 positivity was noted in these RS-like cells seen in the HD-derived SCID tumours.

None of the SCID tumours, like the RS/H cells in the original biopsies expressed any T cell or macrophage -associated antigens, therefore the growth of any residual T cells or macrophages from the injected lymph node suspensions was not supported in our SCID mouse model, although in one case (SCID tumour C1) a very few macrophages were present. The exception was the expression of the CD43 antigen. This antigen was not expressed by the RS/H cells in any HD case, but was highly expressed in all 7 HD-derived SCID tumour. This is a T cell associated antigen but it also marks neoplastic B cells , staining most low grade B cell lymphomas and some high grade B cell lymphomas (Norton & Isaacson 1989). It is also associated with EBV infection and is highly expressed on EBV positive cell lines (Stoll et al 1989).

High expression of the MHC Class II (LN3) served to again demonstrate the activated state of the SCID tumour cells (all HD cases also expressed this antigen), therefore weak CD25 expression was surprising although poor frozen section morphology did effect interpretation some-what.

#### NHL-derived SCID Tumours

This set of SCID tumours, unlike the HD-derived tumours differed histologically and phenotypically.

The SCID tumour J1, derived from an NHL Cb-Cc follicular lymphoma (Case J) did not retain the morphology of the original biopsy instead resembling the HD-derived SCID tumours being a B cell immunoblastic proliferation. The original follicular lymphoma (Case J) expressed high levels of LCA and B cell antigens (CD20, CD19, CD22, MB1 and CD45RA)

being unusual in that no expression of MB2 was seen (Hall et al 1988a, Salter et al 1988, Norton & Isaacson 1989). This SCID tumour expressed various B cell markers quite different to those expressed in the original tumour with some loss of antigen expression in the SCID tumour. However, as with the HD-derived SCID tumours, CD23 (and CD40 ) antigen expression was high (neither being expressed in the original). No T cell markers were expressed but again as with the HD-derived SCID tumours, the CD43 antigen was highly expressed. The activation state of the tumour cells was demonstrated by MHC Class II (LN3) and perhaps CD30 expression. Again CD25 was surprisingly not expressed although the frozen section morphology was poor, but there were a few scanty macrophages present.

In contrast, the SCID tumours derived from the 3 centroblastic lymphomas and the immunoblastic lymphoma - SCID tumours F1 - 14, G1, H1 and I1 appeared to retain the morphology of the original biopsies, although a little more pleomorphic than the originals. Perhaps the length of time growing in the SCID mouse and the lack of host immune intervention allowed tumour progression.

As expected, the 3 centroblastic and immunoblastic lymphomas (Cases F, G, H & I) showed high expression of the LCA (CD45). They were quite unusual in that they were CD19 negative although frozen section morphology made this difficult to interpret in Cases F and I. As expected they all expressed the various B cell antigens (CD20, CD22, MB1, MB2, CD40, CD45RA) in over 50-70% of the tumour cell population and showed no expression of any of the T cell markers (Hall et al 1988a, Salter et al 1988, Norton & Isaacson 1989).

With very few exceptions, these SCID tumours also retained the original tumour phenotype. A notable difference in these SCID tumours was

the lack of expression of the CD23 and CD43 antigens, so heavily expressed in the HD-derived SCID tumours and the J1 SCID tumour. The exception was SCID tumour G1 showing weak CD23 expression however the original biopsy also expressed CD23 weakly. CD23 is expressed in a small proportion of high grade B NHLs (Salter et al 1988). SCID tumour H1 expressed the CD43 antigen, however again this was simply retaining the original phenotype of Case H. CD43 expression is seen in around 10% of high grade B NHL (Norton & Isaacson 1989).

The SCID tumours F1 - F14 from Case F centroblastic lymphoma appeared to retain the phenotype throughout the passages - progressive expression of any one marker was not seen throughout passage.

The 2 LCAL biopsies (Cases K and L) gave rise to 2 morphologically and phenotypically different sets of tumours.

The original LCAL biopsy showed a very restricted antigenic profile. Some, but not all, LCAL express the LCA (Hall et al 1988b). This case did show weak LCA expression but only on frozen section, not on paraffin sections, a pattern recognised previously (Delsol et al 1988, Hall et al 1988b). The LCAL in this study was of B cell type, but the only B cell antigens expressed were MB2 and CD23. These 2 antigens are both found to be expressed in a proportion of LCAL (Herbst et al 1989, Norton & Isaacson 1989). Little data is available on the B cell type of LCAL as they are rare compared to T cell LCAL. In a previous study CD19 and CD22 have been shown to be expressed in B cell LCAL (Herbst et al 1989). However, these 2 B cell markers were negative in the case studied in this thesis. EMA expression was seen in both Cases K and L, an antigen shown to be highly expressed in LCAL (Hall et al 1988b, Norton & Isaacson 1989, Chan et al 1989).



SCID tumours K1 - K4 appeared to be morphologically a 'mixture' of tumour cells resembling those present in the original biopsy and the immunoblastic proliferation with plasmacytoid features as seen in SCID tumour J1 and the HD-derived SCID tumours. This is reflected in the activated B phenotypic profile of these 4 SCID tumours, as apposed to the restricted phenotype of the original biopsy.

In constrast, the SCID tumours from Case L (L1 - L5) tended to retain the morphology and restricted phenotype of the original biopsy, although L1 and L4 both showed an immunoblastic sub-population, this being reflected in the minor expression of LCA and L26 seen with these 2 SCID tumours. The CD30 antigen, expressed by over 90% of LCAL (Norton et al 1989) was surprisingly absent in Cases K and L. However, it is interesting to note that this antigen is highly expressed on all 9 of the LCAL-derived SCID tumours. Perhaps the SCID mouse environment allows expression of this antigen, down-regulated in the original biopsies.

## **Chapter Five**

### **EBV Status of the Original Biopsies and the SCID Tumours**

Because of the known association of the Epstein-Barr virus with HD and NHL, and the fact that inoculation of SCID mice with PBL from EBV-seropositive donors will produce high grade B cell lymphomas, the original HD and NHL biopsies and the resultant SCID tumours were investigated for the presence of EBV to determine if it played a part in these SCID tumours. This was carried out using *in situ* hybridisation for EBER, immunocytochemistry for LMP and EBNA-2, and southern blotting for the presence of the EBV genome. The clonality of any EBV genomes present was also assessed using terminal repeat analysis to determine if any EBV positive SCID tumour was derived from the EBV positive malignant clone in the original biopsy.

The EBV status of the original biopsies and SCID tumours is shown in Table 5.1.

#### **5.1 EBER Expression**

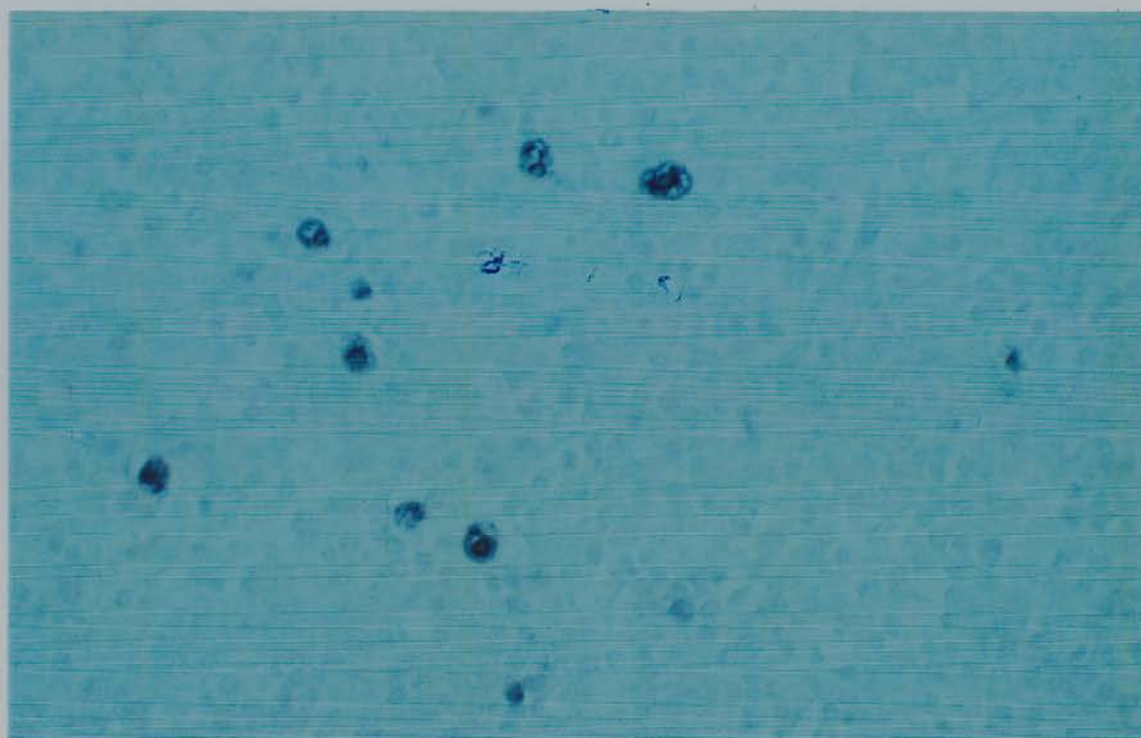
##### **HD Biopsies**

Only 1 of the HD cases - Case E MCHD - showed expression of EBER in the RS/H cells. A strong nuclear signal was seen in the majority if not all of the RS/H cells (Figure 5.1). A small proportion of the surrounding lymphocytes were also EBER positive. In the remaining 4 biopsies, the RS/H cells did not

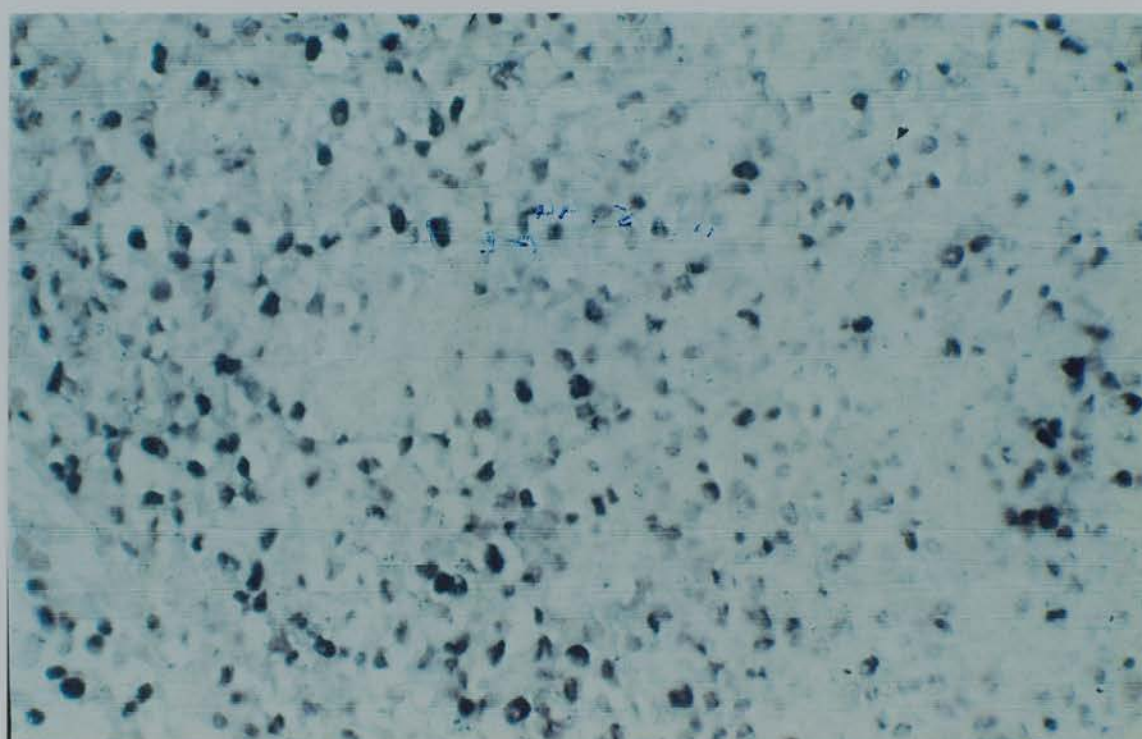
**Table 5.1 EBV Status of the Original Hodgkin's and Non-Hodgkin's Biopsies and SCID Tumours**

ORIGINAL BIOPSY		TRANSPLANTED SCID TUMOUR	
CASE	EBV STATUS	SCID TUMOUR	EBV STATUS
A - LPHD	NEG	A1	EBER/LMP/EBNA-2
B - LPHD	NEG	B1 B2	EBER/LMP/EBNA-2 EBER/LMP/ENBA-2
C - LPHD	NEG	C1 C2	EBER/LMP/EBNA-2 EBER/LMP/EBNA-2
D - NSHD	NEG	D1	EBER/LMP/EBNA-2
E - MCHD	EBER/LMP	E1	EBER/LMP/EBNA-2
F - NHL Cb	NEG	F1 - F14	ALL NEG
G - NHL Cb	NEG	G1	NEG
H - NHL Cb	NEG	H1	NEG
I - NHL ImmB	NEG	I1	NEG
J - NHL Cb/Cc Foll	NEG	J1	EBER/LMP/EBNA-2
K - LCAL	EBER (LMP)	K1 K2 K3 K4	EBER/LMP/EBNA-2 EBER/LMP/EBNA-2 EBER/LMP/EBNA-2 EBER/LMP
L - LCAL	EBER	L1 L2 L3 L4 L5	EBER (LMP/EBNA-2) EBER EBER EBER (LMP/EBNA-2) EBER

Results in brackets indicate minor sub-populations



Case E HDMC, the only EBV positive original HD biopsy showing strong EBER expression in the RS/H cells. Mag x250



HD-derived SCID tumour A1, showing strong EBER expression. Mag x250

**Figure 5.1** EBER Expression in HD Original Biopsy and SCID Tumour

express EBER, only a minority of the back-ground lymphocytes showed expression

#### HD-derived SCID Tumours

All of the HD-derived SCID tumours showed EBER expression. A strong nuclear signal was detected in 50-70% of the tumour cells in each case (Figure 5.1).

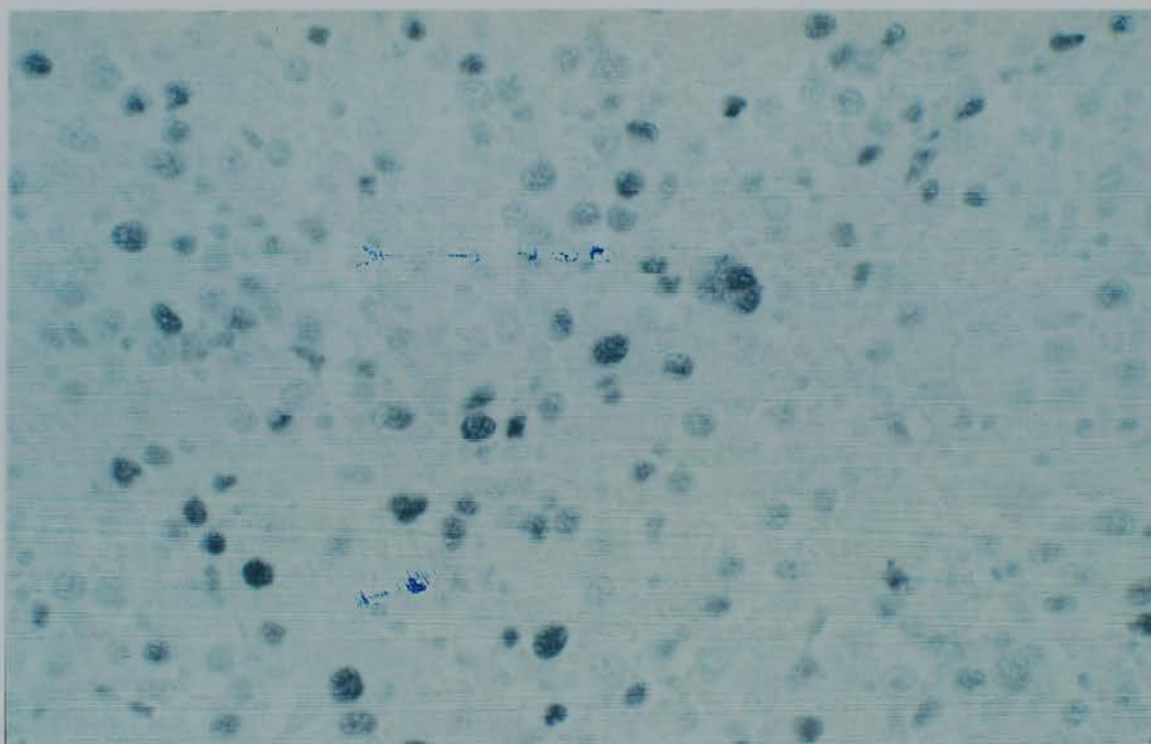
#### NHL Biopsies

The tumour cells in the NHL biopsies F, G, H, I and J were EBER negative. However, in the 2 LCAL biopsies (Cases K and L) the large anaplastic tumour cells were found to show strong nuclear expression of EBER (Figure 5.2). In most cases, a minority population of EBER positive lymphocytes were found.

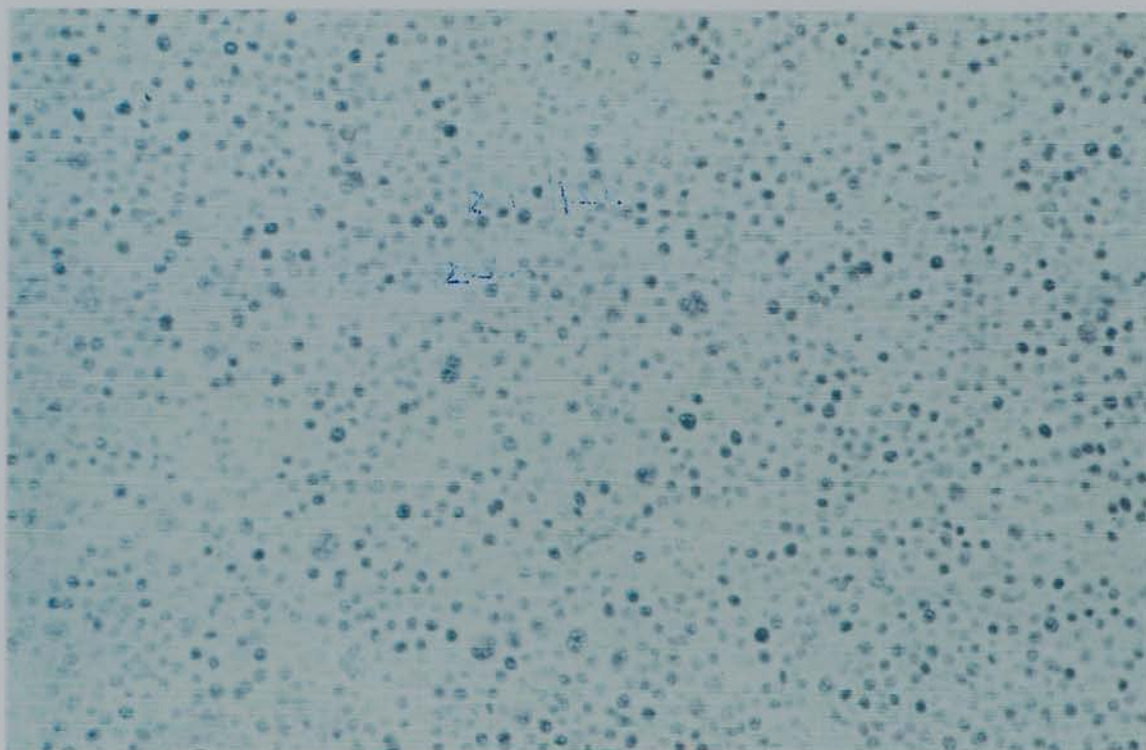
#### NHL-derived SCID Tumours

The SCID tumours derived from Cases F(F1-F14), G(G1), H(H1) and I(I1) were all EBER negative. SCID tumour J1 however showed strong nuclear EBER expression in around 20% of the tumour cell population. The SCID tumours derived from Cases K and L (LCAL) were all found to be EBER positive, retaining the EBER positive phenotype of the original biopsy (Figure 5.2).





Original biopsy Case L - LCAL - showing strong EBER expression in the tumour cells.  
Mag x250



SCID tumour L3 showing strong EBER expression. Mag x100

**Figure 5.2** EBER Expression in Case L - LCAL - and SCID Tumour L3

## **5.2 LMP/EBNA-2 Expression**

### HD Biopsies

In the EBER positive HD biopsy - Case E MCHD - the majority of the RS/H cells were also found to express LMP - EBNA-2 however was not expressed. In the other 4 HD biopsies, the RS/H cells showed no LMP or EBNA-2 expression.

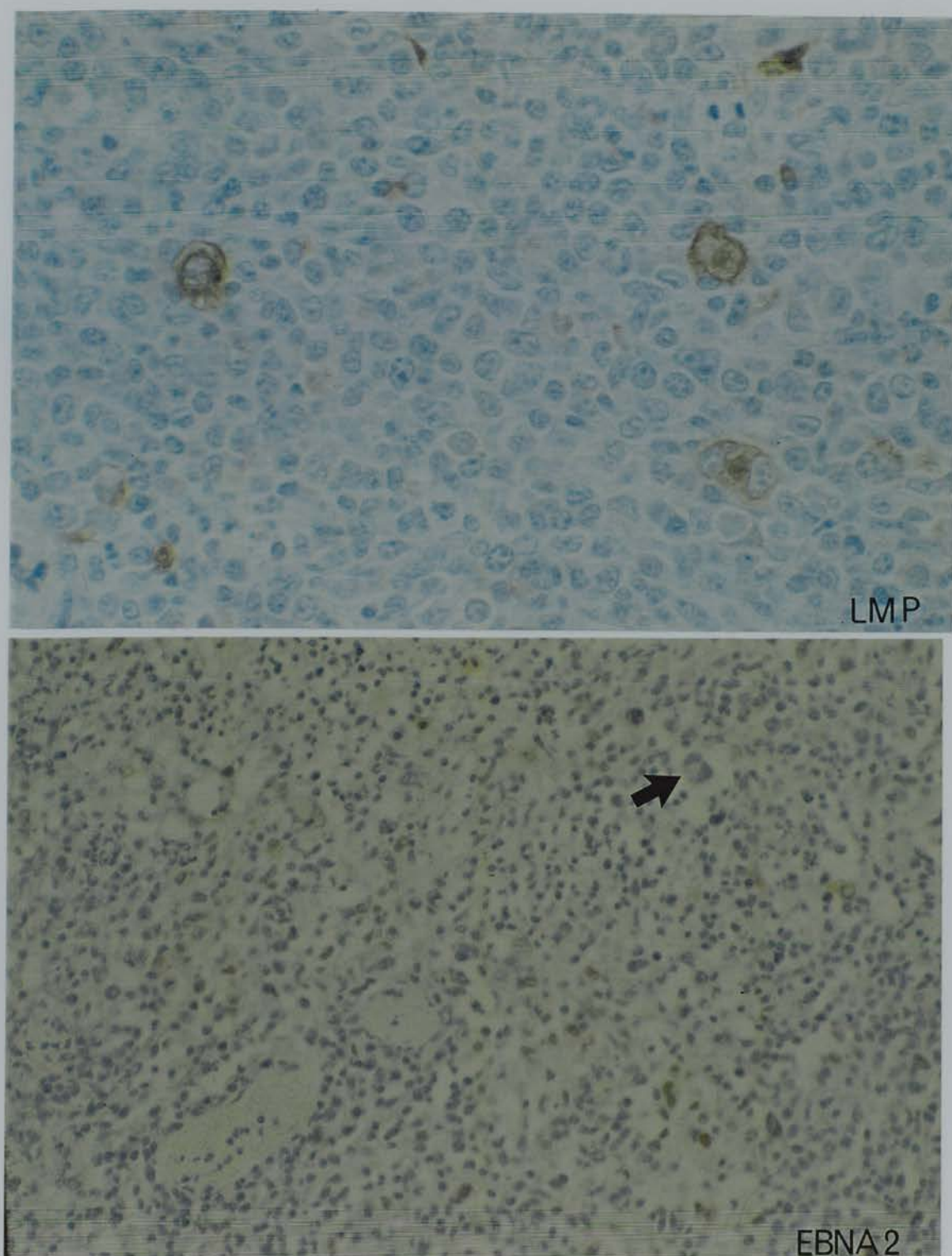
### HD-derived SCID Tumours

All 7 of the HD-derived SCID tumours were found to express both LMP and EBNA-2 - each one tending to show the same staining pattern. Within each SCID tumour population LMP expression was detected in the larger blasts and the multi-nucleated RS-like cells present and in some of the smaller lymphocytes/plasmacytoid cells. EBNA-2 expression was mainly limited to the smaller cells but was present in a proportion of the large blasts i.e. the majority of the larger blasts and RS-like cells had a LMP positive/EBNA-2 negative EBV phenotype while the smaller lymphocytes/plasmacytoid cells and a proportion of the large blasts had a LMP positive/EBNA-2 positive EBV phenotype (Figure 5.3).

30-40% of the large blasts and 5-10% of the smaller blasts in SCID tumour A1 were LMP positive. EBNA-2 expression was seen in 20% of the cell population, mostly in the smaller blasts and plasmacytoid cells, the RS/H-like cells being negative.

In SCID tumour B1, a slightly higher number of cells were LMP positive (around 50%), again showing the pattern of staining as in A1. EBNA-2 expression was seen in 20% of cells, mostly smaller blasts and lymphocytes. The RS/H-like cells were negative. This was also seen in B2 with LMP 30-40% and EBNA-2 10-20%.





**Figure 5.3** LMP/EBNA-2 Expression Pattern of HD-Derived SCID Tumours

The top figure shows LMP expression seen in the large RS/H-like cells of HD-derived SCID tumour B1 (Mag x400), whilst the bottom figure shows a x250 view of EBNA-2 expression from the same case, confined to small blasts and a few large blasts. The multi-nucleated RS-like cell (arrow) is negative.

SCID tumours C1 and C2, again showing the same pattern of staining, showed around 20% of LMP and 10-20% EBNA-2 positive cells.

SCID tumour D1 showed strong LMP expression in the RS/H-like cells and larger blasts (around 40%) and in around 5% of the smaller blasts. EBNA-2 was expressed by 1-5% of the cell population mostly in smaller cells.

SCID tumour E1 showed the same pattern of staining with LMP expressed in 50% and EBNA-2 in 30-40% of the cell population.

### NHL Biopsies

No neoplastic cells in the NHL biopsies showed any expression of LMP or EBNA-2. The exception was Case K LCAL in which a minority of the tumour cells showed LMP but not EBNA-2 expression.

### NHL-derived SCID Tumours

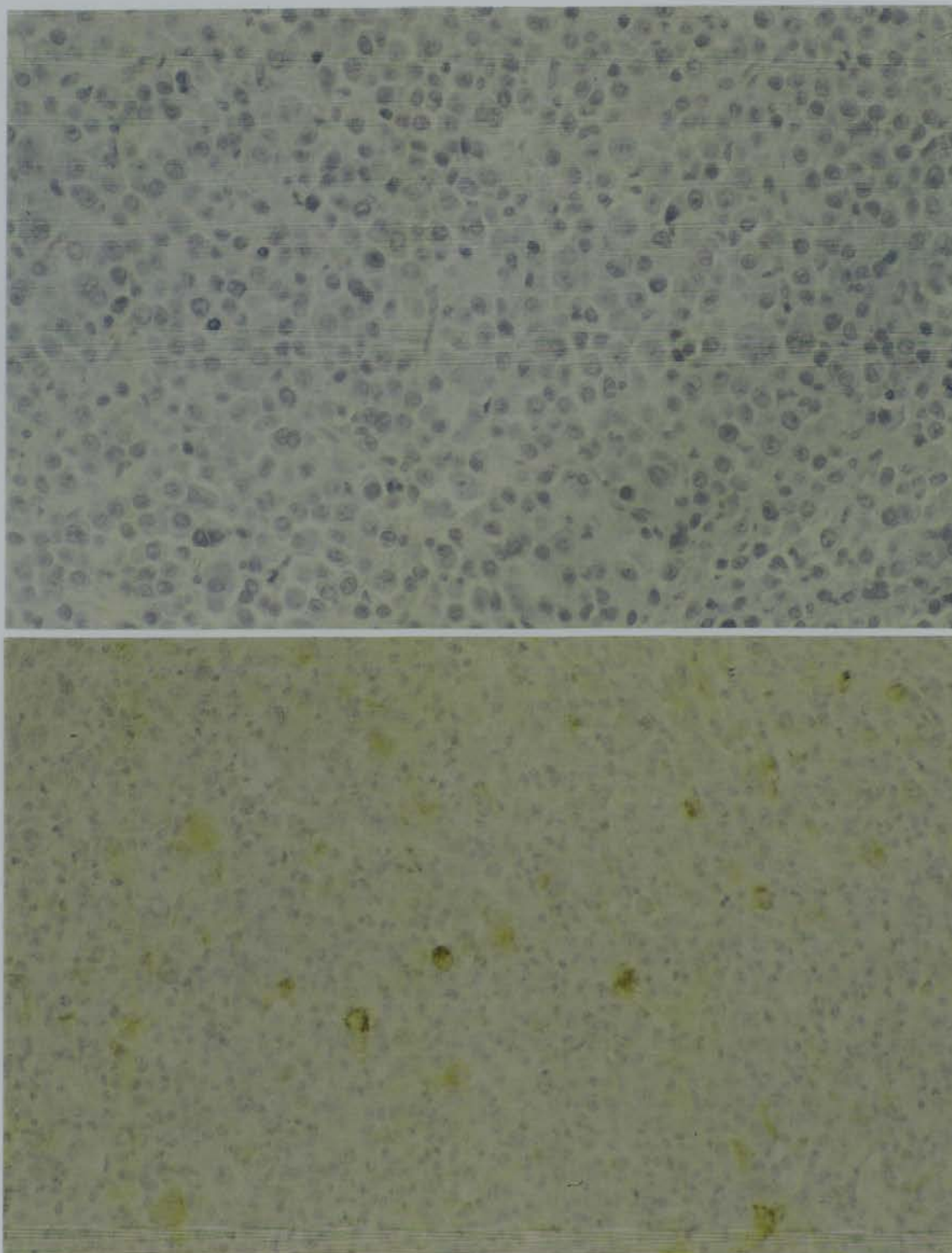
None of the SCID tumours derived from Case F, G, H or I expressed either LMP or EBNA-2.

In SCID tumour J1 however, both LMP and EBNA-2 expression was seen in around 20-30% of the blast cells.

The 2 sets of LCAL-derived SCID tumours (from cases K and L) displayed different EBV phenotypes. The 4 SCID tumours from Case K (K1 - K4) all showed LMP and EBNA-2 expression with the exception of K4 which was LMP positive only. The LMP/EBNA-2 expression was as follows - K1 LMP(20%)/EBNA-2(5-10%), K2 LMP(20%)/EBNA-2 (20%), K3 LMP(10-20%)/EBNA-2(10-20%), K4 LMP(50%).

2 of the SCID tumours derived from Case L LCAL (L3 and L5) were found to be LMP/EBNA-2 negative. SCID tumour L2 showed LMP expression only in a minority population (less than 0.001%). SCID tumour





**Figure 5.4** LMP/EBNA-2 Expression Pattern in SCID Tumour L4

This figure shows the morphological difference between an LMP negative area (top) and an LMP positive area (bottom) of SCID tumour L4. These 2 figures are taken at the same magnification (x250) and clearly show that the large anaplastic cells resembling the original biopsy (Case L) are LMP negative, whilst only focal areas of the smaller immunoblastic cells are LMP positive. The same pattern was seen with EBNA-2.



L1 showed a minority sub-population of LMP+/EBNA-2+ cells (0.01%) - the positive cells were those of the lymphocyte/plasmacytoid sub-population found in L1. The same pattern was seen in SCID tumour L4, although this contained more obvious focal areas of LMP+/EBNA-2+ lymphoproliferations mixed in with the LMP/EBNA-2 negative large anaplastic cells (Figure 5.4).

### **5.3 EBV Detection by Southern Blot Analysis**

This was carried out by Alice Gallagher in the Department of Veterinary Pathology in the University of Glasgow Veterinary School as part of a collaborative study with Dr Ruth Jarrett in whose lab I spent 4 weeks learning the basics of the techniques used.

#### **HD Biopsies**

EBV was also detected in Case E MCHD by Southern blot analysis. Terminal repeat sequence analysis of the EBV genomes was consistent with the presence of a clonal EBV-infected population in this case. EBV was not detected in any of the remaining 4 HD biopsies (Table 5.2).

#### **HD-derived SCID Tumours**

The material from the HD-derived SCID tumours A1, B1 and B2 was not satisfactory for the Southern blot analysis. However, EBV was detected in SCID tumours derived from Cases C, D and E. Terminal repeat sequence analysis was carried out on SCID tumour E1 only, since this was the single case in which the original HD biopsy also showed EBV expression in the RS/H cells. However, the EBV genomes present in the SCID tumour were

**Table 5.2**

**Detection and Clonality of the EBV Genome in  
the Original Hodgkin's Disease Biopsies and  
SCID Tumours Using Southern Blot Analysis**

<b>CASE (HD Biopsy/SCID Tumour)</b>	<b>SOUTHERN BLOT ANALYSIS</b>	
	<b>EBV-SB</b>	<b>EBV-CLONALITY</b>
<b>Case A - LPHD SCID Tumour A1</b>	<b>NEG NS</b>	<b>ND ND</b>
<b>Case B - LPHD SCID Tumour B1 SCID Tumour B2</b>	<b>NEG NS NS</b>	<b>ND ND ND</b>
<b>Case C - LPHD SCID Tumour C1 SCID Tumour C2</b>	<b>NEG POS POS</b>	<b>ND ND ND</b>
<b>Case D - NSHD SCID Tumour D1</b>	<b>NEG POS</b>	<b>ND ND</b>
<b>Case E - MCHD SCID Tumour E1</b>	<b>POS POS</b>	<b>CLONAL OLIGOCLONAL</b>

**KEY:-****NS - Not Satisfactory****ND - Not Done****POS - Positive****NEG - Negative**

found to be oligoclonal and the single clone of EBV-infected cells present in the original Case E was not detected here (Table 5.2).

### NHL Biopsies

The material from Case H centroblastic lymphoma was found to be unsatisfactory for the Southern blot analysis. In NHL cases F, G, H, I and J, the EBV genome was not detected.

However, in both of the LCAL biopsies - Cases K and L - the EBV genome was detected and terminal repeat sequence analysis showed that these 2 biopsies both contained the same single clone of EBV infected tumour cells (Table 5.3).

### NHL-derived SCID Tumours

EBV was not detected in the SCID tumours F1 - F14, G1, H1 and I1. However, it was detected in the J1 SCID tumour. Terminal repeat analysis was not carried out on this case since the original biopsy was EBV negative.

The EBV genome was detected in all of the LCAL-derived SCID tumours (K1 - K4 and L1 - L5). Terminal repeat sequence analysis was carried out on these SCID tumours since EBV was detected in both Cases K and L original biopsies. In each case, a dominant episome was detected i.e. a dominant clone in episomal, closed circular form. However, for each case, linear forms of the virus were also present seen as additional discrete bands on the Southern blot. If the virus is linear, it will give rise to a different sized band even if it is the same EBV. Therefore, where a dominant clone is detected with other bands on the Southern blot, it is difficult to interpret whether these are different EBV clones in smaller populations of cells or if they are linear forms of the dominant clone. No definite conclusion was reached with regard to this, although some of the bands were too small to be

**Table 5.3**

**Detection and Clonality of the EBV Genome  
in the Original Non-Hodgkin's Lymphoma  
Biopsies and SCID Tumours Using Southern  
Blot Analysis**

CASE (NHL Biopsy/SCID Tumour)	SOUTHERN BLOT ANALYSIS	
	EBV-SB	EBV-CLONALITY
Case F - NHL Cb SCID Tumours F1 - F14	NEG NEG	ND ND
Case G - NHL Cb SCID Tumour G1	NEG NEG	ND ND
Case H - NHL Cb SCID Tumour H1	NEG NEG	ND ND
Case I - NHL ImmB SCID Tumour I1	NEG NEG	ND ND
Case J - NHL Cb/Cc Foll SCID Tumour J1	NEG POS	ND ND
Case K - LCAL SCID Tumour K1 SCID Tumour K2 SCID Tumour K3 SCID Tumour K4	POS POS POS POS POS	CLONAL <sup>1</sup> DE+L <sup>1</sup> DE+L <sup>1</sup> DE+L <sup>1</sup> DE+L
Case L - LCAL SCID Tumour L1 SCID Tumour L2 SCID Tumour L3 SCID Tumour L4 SCID Tumour L5	POS POS POS POS POS NS	CLONAL <sup>2</sup> DE+L <sup>2</sup> DE+L <sup>2</sup> DE+L <sup>1</sup> DE+L ND

**KEY**

NEG Negative

POS Positive

ND Not Done

NS Not Satisfactory

DE+L Dominant Episome + Linear Forms

1 Dominant episome is different to the clone in the original biopsy

2 Dominant episome is identical to the clone in the original biopsy



**Figure 5.5 EBV Clonality of Case L - LCAL - and SCID Tumours**

This figure shows the EBV clonality blot of original biopsy Case L - LCAL - and SCID tumours L1, L2, L3 and L4 (L5 DNA was degraded). It demonstrates that the dominant clone of Case L original biopsy is also present in SCID tumours L1, L2 and L3. The dominant clone present in L4 is however different from that of the original biopsy. A very faint band was seen on the original blot in the L4 lane at the same position as the dominant clone of the original biopsy, but unfortunately it was not possible to show this on the photocopy above.



linear forms and therefore this result was interpreted as follows - there is a dominant clone present with other linear forms or episomes present.

However, in the cases of SCID tumours K1, K2, K3 and L4, the dominant clone present was different in each case and different to the clone in the original biopsy (both cases K and L having the same clone of EBV positive cells present as they are sequential biopsies from the same patient). However, in the case of L4, a very faint band was seen in the same position on the Southern blot corresponding to the original biopsy clone. The result is not yet complete on K4.

The dominant clone present in L1, L2 and L3 was however identical to that present in the original biopsy. Unfortunately, L5 tumour material was unsatisfactory for further genotypic analysis (Table 5.3) (Figure 5.5).

## 5.4 Comment

These results show that the Epstein-Barr virus does play a role in a proportion of the SCID tumours. The EBV status of the SCID tumours fell into 3 main groups - a non-restrictive EBV phenotype with expression of a broad range of EBV latent gene products - EBER, LMP and EBNA-2 i.e. a Latency III pattern of infection - this included all 7 of the HD-derived SCID tumours and Cases J and K NHL-derived SCID tumours, a restricted EBV phenotype showing EBER expression only i.e. a Latency I infection pattern - this included the Case L derived SCID tumours, and EBV negative SCID tumours from Cases F, G, H and I. It is assumed here that all the EBV positive cases also expressed EBNA-1, however this was not looked at because of technical problems.

All 7 of the HD-derived SCID tumours expressed EBER, LMP and EBNA-2 in a proportion but not all of the tumour cells - this staining pattern has been noted in similar tumours (Itoh et al 1993). EBV has been shown to be important in the aetiology of HD in a proportion of cases, but only one of our original HD biopsies contained EBV positive RS/H cells (Case E MCHD). However, the SCID tumour derived from this case (E1), as with all of the HD-derived SCID tumours, showed a different EBV latency. The SCID tumour cells expressed EBER, LMP and EBNA-2 (Latency III), not retaining the Latency II EBV infection pattern seen in HD i.e. EBER positive, LMP positive, EBNA-2 negative. There was however a sub-set of large blasts and multi-nucleated RS/H-like cells in these SCID tumours which did follow this Latency II pattern of infection.

Also, Southern blotting analysis demonstrated that whilst the original EBV positive HD biopsy (Case E) contained a single EBV-infected clone of cells, this clone was not present in SCID tumour, suggesting that the SCID tumour was not derived from the EBV-infected clone in the original biopsy. Instead, this E1 SCID tumour was shown to contain oligoclonal EBV genomes consistent with the development of new clones of EBV-transformed B cells.

Therefore, it is a strong possibility that the EBV positive SCID tumours seen after transplantation of HD may simply be secondary to proliferation of normal bystander B cells containing EBV present in the injected lymph nodes rather than proliferation of the neoplastic RS/H cells or their precursors. Most of the original biopsies contained a minority population of EBV positive small lymphocytes. Out-growth of the infected B cells would be controlled by virus-specific cytotoxic T cell immunosurveillance in the immunocompetent human host but once injected into the SCID mouse, the lack of immunosurveillance would allow out-growth of these cells.

It is well documented that normal peripheral blood or tonsillar B lymphocytes from EBV seropositive donors will in most cases produce B cell lymphoproliferations following inoculation into SCID mice (Section 1.6.3)(Cannon et al 1990, Nakamine et al 1991, Rowe et al 1991, Mosier et al 1992, Nadal et al 1992, Purtillo et al 1992, Veronese et al 1994). The EBV positive HD-derived SCID tumours exhibit the same Latency III EBV expression pattern seen in these EBV-induced lymphoproliferations, also seen in LCL and the EBV positive lymphomas associated with immunocompromised individuals.

It was also apparent that the HD-derived SCID tumours were also morphologically similar to these EBV-induced lymphoproliferations being immunoblastic lymphomas with a mixture of small lymphocytes, plasmacytoid differentiation and well-defined areas of necrosis. The HD-derived SCID tumours did show occasional bi-nucleated or multi-nucleated RS-like cells however this morphology could be EBV-induced - RS-like cells have been found in the benign EBV-associated condition infectious mononucleosis (Isaacson et al 1992). Nevertheless, these RS-like cells in the HD-derived SCID tumours did exhibit a Latency II EBV expression pattern seen in HD.

However, reviewing the phenotypic data, it can be seen that the HD-derived SCID tumours showed the same mature, activated B cell phenotype as EBV-transformed B cells (not as the RS/H cells in the original biopsies) with strikingly high expression of the EBV-associated antigens CD23 (Wang et al 1990) and CD43 - an antigen highly expressed on EBV-transformed cell lines (Stoll et al 1989), both being negative in the original biopsy. The CD18 antigen, highly expressed in LCL (reviewed by Middleton et al 1991, Rogers et al 1992) was highly expressed in most but not all of the HD-derived SCID tumours, but the CD43 antigen was a notable feature of the EBV positive SCID tumours, and its expression was obviously not associated with T cells

as no CD3, CD4, CD8 or CD45Ro expression was seen in the SCID tumours. Perhaps the CD30 expression seen in the HD-derived SCID tumours is also a consequence of the EBV 'activated' phenotype (reviewed by Middleton et al 1991, Rogers et al 1992) as opposed to the HD RS cell phenotype seen in the original biopsy, this is supported by CD30 expression in SCID tumours even when the original biopsy RS/H cells were negative.

SCID tumour J1 (derived from follicular lymphoma Case J) shared the Latency III EBV expression pattern seen in the HD-derived SCID tumours, showing expression of EBER, LMP and EBNA-2 even though the tumour cells in the original biopsy were EBV negative. Again, SCID tumour J1 did not retain the morphology and phenotype of the original biopsy but instead (like the HD-derived SCID tumours) resembled the EBV-induced lymphoproliferations described before. Again this tumour showed very high expression of the EBV-associated antigens CD23 and CD43 and it is again a strong possibility that this SCID tumour is simply an out-growth of the EBV-transformed back-ground B cells present in the injected node.

The 2 sets of LCAL-derived SCID tumours exhibited different EBV latency patterns of infection. In the original biopsy, the first biopsy (Case K) was EBER positive with a minority of LMP positive cells. These cells were not present in the second biopsy which was EBER positive only (Latency I). 3 of the SCID tumours derived from Case K - K1, K2 and K3 - all exhibited a Latency III EBV pattern of infection expressing EBER, LMP and EBNA-2, not retaining the original biopsy phenotype. SCID tumour K4 however appeared to retain the original biopsy EBV phenotype (EBER+, LMP+/-). The genotypic analysis showed that all 4 Case K-derived SCID tumours contained a dominant EBV episome but that this was different to the EBV positive clone in the original biopsy. Therefore it appears that these SCID tumours are not derived from the EBV positive malignant clone in the original



biopsy, but from new clones of EBV positive B cells, obviously from expansion of EBV positive bystander B lymphocytes present in the injected lymph node. Perhaps the other bands seen on the Southern blots from these cases are not linear forms of the dominant clone but different sub-clones of EBV-transformed bystander B cells. It is unusual that these 4 SCID tumours showed a degree of morphological similarity to the original biopsy - described as a 'mixture' of large, anaplastic cells similar to the tumour cells in the original biopsy and immunoblast-like cells with some plasmacytoid differentiation. It was initially thought that these K1 - K4 SCID tumours may be a 'mixture' of the original EBER positive malignant clone and new EBV (EBER, LMP, EBNA-2 positive) clones of transformed bystander B cells. However, if this were true, one would expect to see at least a small band on the Southern blot corresponding to the malignant clone in the original biopsy together with the dominant episome present in each K1 - K4 SCID tumour (which was different from the original biopsy). However, the phenotypic analysis also points to an 'activated B ' phenotype with high expression of the EBV-associated antigens CD23 and CD43 seen in the other EBV positive SCID tumours (HD-derived & J1 SCID tumours) not retaining the very restrictive antigenic phenotype of the original biopsy, and it must be concluded at this stage that the K1 - K4 SCID tumours are merely EBV-induced lymphoproliferations derived from the bystander B cell present in the injected lymph node.

Of the 5 SCID tumours derived from Case L (L1 - L5), L3 and L5 showed expression of EBER only, thus retaining the Latency I EBV pattern of expression of the original LCAL biopsy. L1 and L2 also contained a minority sub-population of LMP/EBNA-2 positive and LMP positive immunoblast cells respectively, but the genotypic analysis showed that the dominant EBV episome present in SCID tumours L1, L2 and L3 was identical



to that present in the original biopsy. Therefore it appears that these 3 SCID tumours are derived from the EBER positive malignant clone in the original biopsy. Although this could not be proved for L5, the restricted EBV phenotype of this SCID tumour points to this also being derived from the EBER positive clone in the original biopsy. The minority sub-population of LMP/EBNA-2 positive cells in L1 and L2 may be represented by the other minor bands seen on the southern blot - however, whilst the sub-population present in L1 was seen morphologically and phenotypically (expression of LCA and CD20 was seen in these cells as with the immunoblastic population in the HD-derived and J1 SCID tumours), the LMP positive cells present in L2 were so infrequent (<0.001%), that they were not picked out morphologically by H&E and LCA/CD20 staining was not seen in this SCID tumour. These SCID tumours also retained the morphology and restricted antigenic phenotype of the original biopsy and it is concluded that these 4 SCID tumours (L1, L2, L3, L5) are derived from the malignant clone in the original biopsy with L1 and L2 having a few and a very few respectively new EBV positive B cell clones (from the EBV positive bystander B cells present in the injected lymph node) growing alongside the tumour cells. It is interesting to note that the EBER+ / LMP+/- phenotype of L2 was that seen in the first biopsy of the LCAL (Case K). It is possible that down-regulation of LMP occurred as the tumour progressed (Case L), but the SCID environment allowed expression of this protein again, seen in SCID tumour L2.

SCID tumour L4 appeared to have a similar EBV phenotype to that of L1 i.e. EBER positive with a sub-population of LMP/EBNA-2 positive immunoblasts. However, these immunoblast cells were more common in this tumour with definite focal areas of LMP/EBNA-2 positive cells seen. Indeed, although this SCID tumour contained a dominant episome, this was not the

same as that seen in the other SCID tumours in this group or the original biopsy, although a very faint band was seen at the same position on the Southern blot corresponding to the clone in the original biopsy. This suggests that this SCID tumour L4 is a 'mixture' of the malignant clone of cells from the original biopsy, but the majority are EBV positive immunoblastic cells derived from bystander B lymphocytes present in the injected lymph node.

Again, it is unusual that the morphology of the L4 SCID tumour appeared to suggest that the majority of the SCID tumour cells in L4 were identical to the large, anaplastic cells present in the original biopsy and phenotypically, L4 retained the restricted antigenic phenotype of the original biopsy, only showing LCA and L26 expression in the immunoblastic sub-population. It is therefore surprising that the dominant episome present in the L4 SCID tumour was not that of the malignant clone in the original biopsy - perhaps the block of tissue from this tumour used for DNA analysis contained more focal areas of immunoblastic proliferation than that seen in the block used for histology, this may be possible as quite a bit of variability between different tumour blocks was seen. Therefore, it is concluded at this stage that this SCID tumour cannot be said to represent the original LCAL biopsy.

The SCID tumours derived from the 3 centroblastic lymphomas (Cases F, G and H) and the immunoblastic lymphoma (Case I) were all EBV negative as were the tumour cells in the original biopsies. In contrast to the EBV positive SCID tumours, these tumours retained the morphology and phenotype of the original biopsies and expression of the CD23 and CD43 antigens was not seen here (except when these antigens were expressed in the original biopsies) - further evidence that expression of these antigens is induced by the EBV. Therefore, EBV does not play a part in these tumours

and it appears that they are not merely EBV-induced lymphoproliferations but are derived from the neoplastic cells in the original biopsies.

## **Chapter Six**

### **Immunoglobulin Status of the Original Biopsies and SCID Tumours**

The immunoglobulin (Ig) expression, as determined by immunocytochemistry, of the SCID tumours was investigated to determine how this compared to the original biopsy, and to determine the clonality of the SCID tumours. Clonality was also investigated using Ig gene rearrangement analysis. These techniques were used to compare clonal populations present in the original and SCID tumours to determine whether or not the SCID tumours were derived from the malignant clone in the original biopsy. Because of the involvement of the Epstein-Barr virus in a proportion of these SCID tumours, Ig secretion - a feature of EBV-immortalised B cells - was investigated.

#### **6.1 Immunoglobulin Expression**

##### **HD Biopsies**

The RS/H cells in each of the original HD biopsies were found to show diffuse cytoplasmic staining of polytypic immunoglobulin (Ig), always both kappa and lambda light chain staining was seen in each case. Therefore, this is shown in Table 6.1 simply as non-specific absorption.

The Ig staining pattern observed in the NHL biopsies and the SCID tumours was usually dense cytoplasmic and membrane staining with some granular golgi and peri-nuclear clustering indicative of specific Ig synthesis. Interpretation of the Ig staining was carried out with caution as a lot of background staining was seen in some cases because of the presence of inter-cellular Ig 'sticking' non-specifically to surrounding cells or connective tissue.

#### HD-Derived SCID Tumours

Ig expression of the HD-derived SCID tumours is shown in Table 6.1. All of the HD-derived SCID tumours expressed both heavy and light chain immunoglobulin. These tumours were not monoclonal in nature, but there appeared to be a marked predominance of a single heavy and a single light chain indicating a dominant clone in most cases. The dominant heavy and light chain comprised between 50-70% of the large immunoblasts and plasmacytoid cells, with the sub-clones comprising 2-5% of the tumour cell population - Figure 6.1 shows the Ig phenotype of SCID tumour A1.

The SCID tumour B1 showed a dominant clone of IgA, $\lambda$  positive tumour cells with a minor population expressing kappa. However, the passaged B1 tumour (SCID tumour B2) only showed the growth of the dominant clone IgA, $\lambda$ .

SCID tumours C1 and C2 did not show a predominance of a single Ig heavy and light chain, however it was still possible to distinguish a higher expression of IgM over IgG and lambda over kappa in the case of C1 and kappa over lambda in the case of C2.

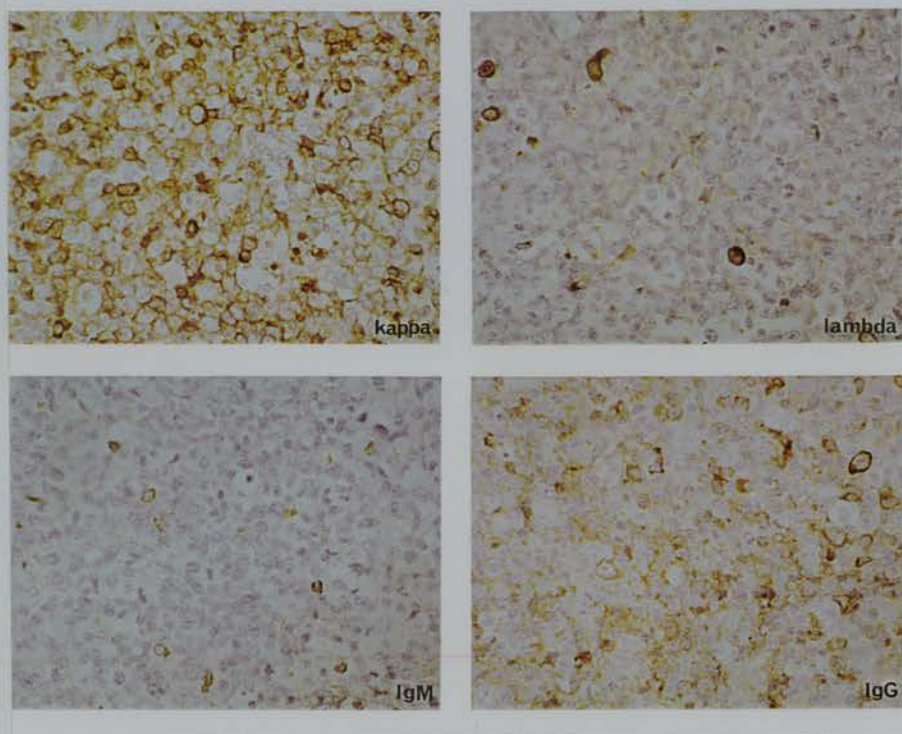


Table 6.1

Immunoglobulin Expression of Original Hodgkin's Disease Biopsies and SCID Tumours Using Immunocytochemical Analysis

CASE (HD Biopsy/SCID Tumours)	Ig ISOTYPE EXPRESSION
Case A - LPHD SCID Tumour A1	non-specific absorption IgG, $\kappa$ (M,A, $\lambda$ )
Case B - LPHD SCID Tumour B1 SCID Tumour B2	non-specific absorption IgA, $\lambda$ ( $\kappa$ ) IgA, $\lambda$
Case C - LPHD SCID Tumour C1 SCID Tumour C2	non-specific absorption IgM,G, $\lambda$ , $\kappa$ M>G / $\lambda$ > $\kappa$ IgG, $\kappa$ , $\lambda$ (M,A) $\kappa$ > $\lambda$
Case D - NSHD SCID Tumour D1	non-specific absorption IgG, $\lambda$ (M, $\kappa$ )
Case E - MCHD SCID Tumour E1	non-specific absorption IgM, $\kappa$ (G, $\lambda$ )

Results in brackets indicate minor sub-populations



**Figure 6.1** Immunoglobulin Expression of SCID Tumour A1

This figure shows the dominant Ig heavy and light chain expression nature of the SCID tumours - in this case IgG, $\kappa$  (M,  $\lambda$ ) - IgA was as the IgM and lambda.

### NHL Biopsies

The Ig expression of the tumour cells in the NHL biopsies is shown in Table 6.2. The tumour cell population in all of the NHL biopsies showed monoclonal Ig expression. All cases showed both Ig heavy and light chain expression except the LCAL which only expressed the IgA heavy chain, showing no light chain expression.

### NHL-Derived SCID Tumours

The Ig expression of the NHL-derived SCID tumours is shown in Table 6.2. SCID tumour J1, like the HD-derived SCID tumours, was a polyclonal tumour. However, again it showed a predominance of a single Ig heavy and light chain - in this case IgG and Kappa expression with a sub-population of IgM, A and lambda expressing cells - different from the original IgG, $\lambda$  follicular lymphoma (Case J).

In contrast, the SCID tumours derived from Cases F, G, H and I showed monoclonal Ig expression identical to that seen in the original biopsies. The Ig phenotype (IgM, $\kappa$ ) was retained throughout the passages of the SCID tumours derived from Case F.

The 2 sets of SCID tumours derived from the LCAL (Cases K and L) again showed very different properties. The Ig expression of the 4 SCID tumours derived from Case K (K1-K4) resembled that of the HD-derived SCID tumours and SCID tumour J1. Again these were polyclonal tumours but again showed a predominant expression of a single Ig heavy and light chain. However, none of the 4 SCID tumours K1-K4 showed predominant IgA expression like the original biopsy, although K4 contained an IgA-expressing sub-population. SCID tumour K3 was the exception in that it only showed expression of IgG and kappa.

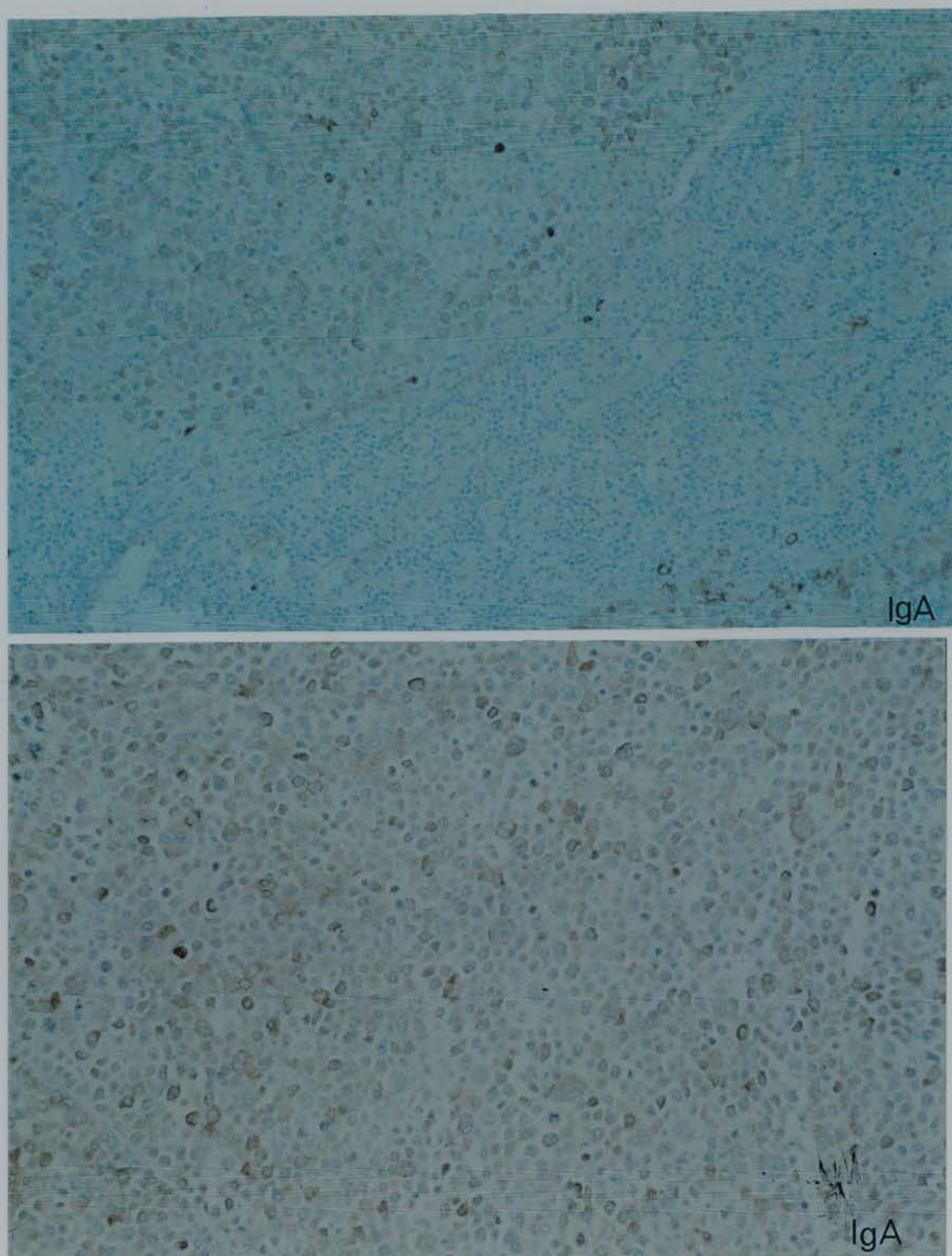
**Table 6.2**

**Immunoglobulin Expression of Original  
Non-Hodgkin's Lymphomas  
Biopsies and SCID Tumours Using  
Immunocytochemical Analysis**

<b>CASE (NHL Biopsy/SCID Tumours)</b>	<b>Ig ISOTYPE EXPRESSION</b>
<b>Case F - NHL Cb SCID Tumours F1 - F14</b>	<b>IgM,<math>\kappa</math> all IgM,<math>\kappa</math></b>
<b>Case G - NHL Cb SCID Tumour G1</b>	<b>IgM,<math>\kappa</math> IgM,<math>\kappa</math></b>
<b>Case H - NHL Cb SCID Tumour H1</b>	<b>IgG,<math>\lambda</math> IgG,<math>\lambda</math></b>
<b>Case I - NHL ImmB SCID Tumour I1</b>	<b>IgG,<math>\kappa</math> IgG,<math>\kappa</math></b>
<b>Case J - NHL Cb/Cc Foll SCID Tumour J1</b>	<b>IgG,<math>\lambda</math> IgG,<math>\kappa</math> (M,A,<math>\lambda</math>)</b>
<b>Case K - LCAL SCID Tumour K1 SCID Tumour K2 SCID Tumour K3 SCID Tumour K4</b>	<b>IgA IgG,<math>\lambda</math> (M) IgM,<math>\lambda</math> (G,<math>\kappa</math>) IgG,<math>\kappa</math> IgM,<math>\kappa</math>,<math>\lambda</math> (G,A)</b>
<b>Case L - LCAL SCID Tumour L1 SCID Tumour L2 SCID Tumour L3 SCID Tumour L4 SCID Tumour L5</b>	<b>IgA IgA (M,<math>\lambda</math>) IgA IgA IgA (M,<math>\kappa</math>) IgA</b>

**Results in brackets indicate a minor sub-population**





**Figure 6.2** Immunoglobulin Expression of Original LCAL Biopsy Case L and SCID Tumour

IgA expression is seen in the tumour cells of the original biopsy Case L (top) - this Ig phenotype was retained in the SCID tumours L1, L2, L3 & L5. L2 is shown in the bottom photograph. Mag x100



The 5 SCID tumours derived from Case L (L1-L5) all showed identical Ig expression to that seen in the original biopsy (IgA) (Figure 6.2). However, SCID tumour L1 also contained a sub-population of IgM, $\lambda$  expressing cells. In the case of L4, an IgM, $\kappa$  positive sub-population was also present although more evident than the sub-population in L1. These cells were not the large, anaplastic tumour cells seen in Case L and SCID tumours L1-L5 (they expressed IgA only), the positive cells were of the immunoblastic sub-population found in L1 and L4.

## **6.2 Immunoglobulin Gene Rearrangements**

Both PCR and Southern blotting were used for detection of Ig gene rearrangements. This is because PCR only picks out around 80% of IgH gene rearrangements so some are missed by this technique. Therefore, southern blotting was carried out in these cases indicated in the text.

This was carried out by Alice Gallagher in the Department of Veterinary Pathology in the University of Glasgow Veterinary School as part of our collaborative study with Dr Ruth Jarrett.

The Ig gene rearrangement results for the HD and NHL original biopsies and the resultant SCID tumours are presented in Tables 6.3 and 6.4.

### **HD Biopsies**

No IgH gene rearrangements were detected in any of the original HD biopsies by PCR. It was decided that there was no need to carry out Southern blotting for IgH gene rearrangements on these cases since none

**Table 6.3**

**Immunoglobulin Gene Rearrangements in  
the Original Hodgkin's Disease Biopsies and  
SCID Tumours Detected by PCR**

<b>CASE (HD Biopsy/SCID Tumour)</b>	<b>Ig GENE REARRANGEMENT ANALYSIS</b>	
	<b>Ig-PCR</b>	<b>Notes</b>
<b>Case A - LPHD SCID Tumour A1</b>	<b>No IgR</b>	<b>- clonal</b>
<b>Case B - LPHD SCID Tumour B1 SCID Tumour B2</b>	<b>No IgR NS</b>	<b>- clonal -</b>
<b>Case C - LPHD SCID Tumour C1 SCID Tumour C2</b>	<b>No IgR IgR</b>	<b>- clonal clonal C1 &amp; C2 IgR different</b>
<b>Case D - NSHD SCID Tumour D1</b>	<b>No IgR</b>	<b>- clonal</b>
<b>Case E - MCHD SCID Tumour E1</b>	<b>No IgR</b>	<b>- clonal</b>

**KEY**

**IgR**    Immunoglobulin gene rearrangement was found  
**NS**    Not satisfactory (insufficient material or DNA degradation)

**Table 6.4**

**Immunoglobulin Gene Rearrangements in  
the Original Non-Hodgkin's Lymphoma  
Biopsies and SCID Tumours Detected by  
PCR and Southern Blot Analysis**

<b>CASE (NHL Biopsy/SCID Tumour)</b>	<b>Ig GENE REARRANGEMENT ANALYSIS</b>	
	<b>IgR-PCR</b>	<b>NOTES</b>
<b>Case F - NHL Cb SCID Tumours F1 - F14</b>	<b>clonal IgR all clonal IgR</b>	<b>IgR all identical</b>
<b>Case G - NHL Cb SCID Tumour G1</b>	<b>clonal IgR clonal IgR</b>	<b>- identical to original</b>
<b>Case H - NHL Cb SCID Tumour H1</b>	<b>clonal IgR NS</b>	<b>- -</b>
<b>Case I - NHL ImmB SCID Tumour I1</b>	<b>NEG with PCR NEG with PCR</b>	<b>Case I DNA degraded (SB not done) I1 - clonal IgR with SB</b>
<b>Case J - NHL Cb/Cc Foll SCID Tumour J1</b>	<b>NEG with PCR clonal IgR</b>	<b>SB not done</b>
<b>Case K - LCAL SCID Tumour K1 SCID Tumour K2 SCID Tumour K3 SCID Tumour K4</b>	<b>NS clonal IgR clonal IgR clonal IgR NC</b>	<b>- all IgR different and different from Case L original biopsy</b>
<b>Case L - LCAL SCID Tumour L1 SCID Tumour L2 SCID Tumour L3 SCID Tumour L4 SCID Tumour L5</b>	<b>clonal IgR clonal IgR clonal IgR clonal IgR clonal IgR NS</b>	<b>All detected by SB L1, L2 and L3 IgR all identical to original biopsy L4 IgR different from original biopsy</b>

**key**

**IgR** Immunoglobulin gene rearrangement detected  
**NS** not satisfactory (insufficient material or DNA degradation)  
**NC** result not completed yet  
**SB** southern blot analysis

were detected by PCR even though the SCID tumours derived from these cases all has detectable IgH rearrangements by this method.

#### HD-derived SCID Tumours

The material from SCID tumour B2 was found to be unsatisfactory for genotyping.

A clonal IgH gene rearrangement was found in the remaining HD-derived SCID tumours. The 2 primary SCID tumours derived from Case C (LPHD) were found to exhibit different clonal IgH gene rearrangements.

#### NHL Biopsies

A clonal IgH gene rearrangement was detected in Cases F, G and H. In Case I biopsy, no DNA was left for Southern blot analysis - PCR being unable to detect an IgH gene rearrangement. In Case J, no IgH gene rearrangement was detected by PCR, but Southern blotting was not carried out as an IgH gene rearrangement was detected in the J1 SCID tumour by PCR. There was insufficient material available from Case K for IgH gene rearrangement studies, however a clonal IgH gene rearrangement was detected in Case L using Southern blotting and all the SCID tumours (i.e. from both K1 -K4 and L1 - L5) rearrangement analysis was compared to this since Case K and Case L are sequential biopsies from the same patient.

#### NHL-derived SCID Tumours

An identical clonal IgH gene rearrangement was detected in all of the F1 - F14 SCID tumours and this rearrangement was identical to that present in the original Case F biopsy. This was also seen in SCID tumour G1, in which the clonal IgH gene rearrangement detected was identical to that present in the original Case G biopsy.

Unfortunately, the DNA from SCID tumour H1 was not suitable for any genotypic analysis, therefore comparisons of IgH rearrangements between this SCID tumour and the original Case H biopsy were not possible.

No Ig rearrangement was detected in SCID tumour I1 by PCR, but a clonal IgH gene rearrangement was detected using Southern blotting. A clonal IgH gene rearrangement was detected in SCID tumour J1 by PCR.

Clonal IgH gene rearrangements were detected in SCID tumours K1, K2 and K3 (K4 result is not available at the moment) by PCR, but these were all different rearrangements and different from that detected in the original biopsy. However, the clonal IgH gene rearrangements detected in SCID tumours L1, L2 and L3 were identical and identical to that present in the original biopsy. However, the clonal IgH gene rearrangement detected in SCID tumour L4 was not the same as that in the original biopsy. Unfortunately, the DNA available from SCID tumour L5 was insufficient for Southern blotting analysis.

### **6.3 SCID Tumour Immunoglobulin Secretion**

The aim of investigating the Ig secretion was not to quantitate the amount of Ig secreted by the SCID tumours, but to determine if there was a link between the EBV status of the SCID tumours and Ig secretion (a feature of EBV-immortalised B cells). Therefore, this was not carried out in a quantitative manner, although the separate Ig isotypes were looked at for each Ig secretor case.

Blood was taken from every SCID mouse at the post-mortem examination, whether or not they were tumour-bearing mice. Each was tested for the presence of human Ig and murine Ig (to check if they were



'leaky'). No tumour-free mouse exhibited any trace of human Ig in their serum. However, of these non-tumour mice, 6% were found to contain murine Ig in their serum, although always of a much lower level than that found in normal mouse serum.

#### HD-Derived SCID Tumours

All 7 of the HD-derived SCID tumours showed secretion of human Ig. The OD readings are given in Table 6.5. The readings shown are those obtained after subtracting the back-ground reading given with normal SCID serum (NSS). In column 7, the OD results are presented in a semi-quantitative way as a means of summary of the OD readings. This matches the way that the Ig expression (by immunohistochemistry) results were presented, with the results in brackets indicating 'slight' secretion. It should be noted that each tumour secreted very different levels of Ig, and what OD result may appear as slight secretion for one tumour, may appear as one of the main Ig isotypes secreted in another. For example, an IgA OD reading of 0.093 is classed as slight secretion in SCID tumour A1, but a kappa OD reading of 0.091 is one of the main Ig isotypes secreted in SCID tumour E1 because of the over-all lower Ig secretion in that tumour. Therefore, the Ig secretion 'summary' results given in column 7 cannot really be compared between different SCID tumours. The reason for different over-all levels of Ig secretion could be a consequence of different-sized SCID tumours - it is interesting to note that SCID tumours B1 and B2 (passage of B1) secrete identical main Ig isotypes, but B2 in much smaller quantities - this B2 SCID tumour was much smaller than B1, so small it was inadequate for further passage. Also, SCID tumours C1 and C2 were bigger than the other HD-

Table 6.5

**Optical Density Readings of Immunoglobulin Secretion from SCID Tumours**

SAMPLE	OD READING AT 490nm MINUS BACK-GROUND (NSS OD)					
	IgG	IgM	IgA	kappa	lambda	Summary
NSS	0.013	0.013	0.011	0.004	0.019	-
NHS	0.593	0.176	0.200	0.218	0.424	-
SCID Tumour A1	0.134	0.125	0.093	0.125	0.070	IgG, $\kappa$ ,M (A, $\lambda$ )
SCID Tumour B1	0.005	0.016	0.259	0.007	0.228	IgA, $\lambda$
SCID Tumour B2	0.008	0.001	0.092	0	0.062	IgA, $\lambda$
SCID Tumour C1	0.377	0.308	0.047	0.204	0.251	IgM,G, $\lambda$ , $\kappa$
SCID Tumour C2	0.552	0.172	0.081	0.042	0.550	IgG, $\lambda$ ,M
SCID Tumour D1	0.288	0.207	-0.002	0.068	0.403	IgG, $\lambda$ ,M ( $\kappa$ )
SCID Tumour E1	0.041	0.082	0.013	0.091	0.043	IgM, $\kappa$ (G, $\lambda$ )
SCID Tumour I1	0.075	-0.008	0.003	0.018	0.011	IgG
SCID Tumour K1	0.423	0.080	0.001	0.017	0.363	IgG, $\lambda$ (M)
SCID Tumour K2	0.092	0.115	0.004	0.016	0.097	IgM, $\lambda$ ,G
SCID Tumour K3	0.084	0.014	0.010	0.048	0.037	IgG, $\kappa$ , $\lambda$
SCID Tumour L4	0.030	0.137	0.002	0.060	0.007	IgM ( $\kappa$ )

**key**

NSS Normal SCID Serum

NHS Normal Human Serum

Results in brackets indicate minor isotypes

Table 6.6

**Immunoglobulin Expression and Secretion  
of SCID Tumours - Correlation with EBV Status**

SCID TUMOUR	EBV STATUS	Ig ISOTYPE EXPRESSION	Ig ISOTYPE SECRETION (ELISA)
A1	POS	IgG, $\kappa$ (M,A, $\lambda$ )	IgG, $\kappa$ ,M (A, $\lambda$ )
B1 B2	POS POS	IgA, $\lambda$ ( $\kappa$ ) IgA, $\lambda$	IgA, $\lambda$ IgA, $\lambda$
C1 C2	POS POS	IgM,G, $\lambda$ , $\kappa$ IgG, $\kappa$ , $\lambda$ (M,A)	IgM,G, $\lambda$ , $\kappa$ IgG, $\lambda$ ,M
D1	POS	IgG, $\lambda$ (M, $\kappa$ )	IgG, $\lambda$ ,M ( $\kappa$ )
E1	POS	IgM, $\kappa$ (G, $\lambda$ )	IgM, $\kappa$ (G, $\lambda$ )
F1 - F14	ALL NEG	ALL IgM, $\kappa$	NON-SECRETORS
G1	NEG	IgM, $\kappa$	NON-SECRETOR
H1	NEG	IgG, $\lambda$	NON-SECRETOR
I1	NEG	IgG, $\kappa$	IgG
J1	POS	IgG, $\kappa$ (M,A, $\lambda$ )	NO BLOOD
K1 K2 K3 K4	POS POS POS POS	IgG, $\lambda$ (M) IgM, $\lambda$ (G, $\kappa$ ) IgG, $\kappa$ IgM, $\kappa$ , $\lambda$ (G,A)	IgG, $\lambda$ (M) IgM, $\lambda$ ,G IgG, $\kappa$ , $\lambda$ NO BLOOD
L1 L2 L3 L4 L5	EBER(LMP/EBNA-2) EBER EBER EBER(LMP/ENBA-2) EBER	IgA (M, $\lambda$ ) IgA IgA IgA (M, $\kappa$ ) IgA	NON-SECRETOR NON-SECRETOR NON-SECRETOR IgM ( $\kappa$ ) NON-SECRETOR

Results in brackets indicate minor sub-populations or isotypes

derived SCID tumours and these SCID tumours both secrete more Ig as a whole.

However, as Table 6.6 shows, the specific Ig isotypes secreted by each SCID tumour can be compared to the specific Ig isotypes expressed by the tumour cells as determined by immunohistochemistry, and these correlate very well. The dominant heavy and light chain present in each HD-derived SCID tumour were also the dominant isotypes secreted in each case, although in some cases (SCID tumours A1 and D1) one of the Ig chains only found in a minor population was secreted in almost equal quantities to that of the predominant Ig isotypes, and in another case (SCID tumour C2) one of the dominant isotypes present was not secreted (kappa).

#### NHL-Derived SCID Tumours

The SCID tumours derived from Cases F, G and H did not show any Ig secretion. No blood was available for Case J. Ig secretion was seen in SCID tumours I1, K1, K2, K3 (no blood was available for K4) and from L4. The OD results for these tumours are given in Table 6.5 - the results are presented as for the HD-derived SCID tumours. Again, as can be seen from Table 6.6, the specific Ig isotypes secreted by the SCID tumours correlate very well to the Ig isotypes expressed as determined by immunohistochemistry.

As can be seen from Table 6.6, all of the EBV (EBER, LMP, EBNA-2) positive SCID tumours showed secretion of Ig. The EBER positive only SCID tumours (L2, L3 and L5) were all non-secretors as was L1 which has only a sub-population of LMP/EBNA-2 positive cells. SCID tumour L4, in

which focal LMP/EBNA-2 positive expression was seen, showed secretion of Ig. These LMP/EBNA-2 positive cells seen within SCID tumour L4 were the IgM, $\kappa$  positive immunoblasts, and it was only this isotype of Ig that was secreted - the IgA isotype seen in the large anaplastic cells also present in this tumour was not secreted.

None of the EBV negative SCID tumours secreted Ig except SCID tumour I1 which secreted some IgG.

## **6.4 Comment**

Although Ig light chain restriction has been reported in LPHD (Schmid et al 1991a, Momose et al 1992), all of the original HD biopsies here showed non-specific poly-typic Ig staining, presumably via endocytosis of exogenous immunoglobulin. Therefore the malignant RS/H cells in the original biopsies did not appear to be show specific expression of any Ig heavy or light chain. However, all 7 of the HD-derived SCID tumours did show expression of both heavy and light chain Ig, therefore not retaining the phenotype of the original biopsies. None of the HD-derived SCID tumours were monoclonal in nature using immunohistochemical analysis, although in each case a predominance of a single heavy and light chain was seen. This would appear to indicate that these SCID tumours are comprised of a dominant clone and indeed, passage seemed to favour the growth selection of this clone - SCID tumour B2 (the passaged B1) was comprised only of the IgA, $\lambda$  dominant clone seen in SCID tumour B1. This 'dominant clone' nature was confirmed by the genotypic analysis of these SCID tumours - all 7 of the HD-derived SCID tumours showed clonal IgH gene rearrangements, although this method was perhaps not sensitive enough to detect the sub-clones present in each case.



Unfortunately, no IgH gene rearrangements were detectable in any of the HD biopsies, therefore comparison of Ig clonality was not possible. Therefore, no definite conclusions can be drawn from the Ig analysis alone on whether or not the dominant clone present in the HD-derived SCID tumours is derived from the malignant clone in the original HD biopsy, although the differences in Ig expression between the original biopsies and the SCID tumours detected by immunohistochemical analysis and the EBV positive nature of these SCID tumours makes this highly unlikely.

It appears that a single dominant EBV-transformed B cell clone is present in each HD-derived SCID tumour - this has also been described in the EBV positive SCID tumours derived from inoculation of PBL from EBV sero-positive donors and in similar EBV-induced lymphoproliferative disease in SCID mice (Rowe et al 1991, Nakamine et al 1993). These monoclonal EBV positive SCID tumours were found to contain the normal diploid DNA content and to exhibit no abnormal chromosomal translocations (Rowe et al 1991). Therefore, it appears that the 'dominant clone' nature of these HD-derived SCID tumours is not suggestive of malignant transformation, but a selection of a dominant EBV positive B cell clone based on growth rate. It is well known that even during EBV-induced B cell immortalisation of lymphoblastoid cell lines (LCL) *in vitro*, where there is no obvious selection except on the basis of growth rate, the original polyclonal LCL can soon be dominated by one or a few clones with serial passage in culture (Bechet et al 1974).

Further evidence that these SCID tumours were not derived from the malignant clone in the original HD biopsy is that the 2 primary SCID tumours derived from Case C LPHD showed different clonal IgH gene rearrangements from each other.

A similar pattern was observed in the other EBV (EBER/LMP/EBNA-2) positive SCID tumours (J1, K1, K2, K3, K4), each expressing a dominant single heavy and light Ig chain. Again, in these cases, comparison of Ig expression detected by immunocytochemical analysis, demonstrated that the dominant clone present in these J1, K1, K2, K3 and K4 SCID tumours did not match that of the original biopsies. IgH gene rearrangement analysis supports this in Case J - a rearrangement not being detected here by PCR but detectable in the SCID tumour J1. This suggests that the rearrangement in the original Case J was one of the 20% not picked up by PCR, the fact that a rearrangement was picked up in the SCID tumour J1 by PCR indicates that the rearrangement in the SCID tumour is not identical to that in the original biopsy. Although DNA analysis is not yet complete for K4, IgH gene rearrangement analysis of K1, K2 and K3 showed that these SCID tumours all had different IgH gene rearrangements from their original biopsies, demonstrating that these SCID tumours are not derived from the malignant clone in the original biopsies and the 'dominant clone' nature of these SCID tumours is as for the HD-derived SCID tumours.

However, the set of 5 SCID tumours derived from Case L - LCAL displayed different Ig characteristics to that of the 4 Case K-derived SCID tumours. Case L (and previous biopsy Case K) was unusual in that it only expressed the  $\alpha$  heavy chain with no light chain expression, a consequence of neoplasia. This isolated heavy chain expression, although rare, has been noted in NHL before (Gorden 1984) and may be caused by a defect in ability to carry out light chain gene rearrangements, or rearrangements on all light chain alleles may have occurred but none were productive.

SCID tumours L1, L3 and L5 all showed identical Ig expression to the original biopsy, retaining this isolated IgA expression of the large anaplastic cells, and the genotypic analysis from L1 and L3 confirmed that these SCID

tumours contained an identical IgH gene rearrangement to the original biopsy (unfortunately the L5 SCID tumour sample was inadequate for DNA analysis). This confirms the EBV clonality results, demonstrating that these SCID tumours are derived from the malignant clone in the original biopsy.

SCID tumour L1, although the majority population of large anaplastic cells present showed identical Ig expression to the original biopsy, the LMP/EBNA-2 positive minority sub-population of immunoblastic cells were evidenced by IgM, $\lambda$  expression. However, as with the EBV clonality analysis, this sub-population was dismissable as the Ig analysis demonstrated that this SCID tumour also showed an identical IgH gene rearrangement to that seen in the original biopsy.

The SCID tumour L4, using immunohistochemical analysis, appeared to be a 'mixture' of IgA expressing large anaplastic cells as the tumour cells in the original biopsy and IgM, $\kappa$  expressing immunoblastic cells - the LMP/EBNA-2 positive population present in this SCID tumour. The Ig rearrangement analysis also confirmed the EBV clonality analysis on this L4 SCID tumour - the IgH gene rearrangement found in L4 was different to that found in the original biopsy, indicating that this SCID tumour was not derived from the malignant clone in the original biopsy. Perhaps the IgH rearrangement present in this SCID tumour was that of the IgM, $\kappa$  expressing immunoblast cell population. This result again seems unusual since it appeared morphologically and phenotypically that the major cell population present in this SCID tumour was the IgA expressing large anaplastic cells identical to those present in the original biopsy. However, both the EBV clonality and the IgH gene rearrangement analyses lead to the conclusion that this SCID tumour cannot be said to represent the original LCAL biopsy.

In the remaining NHL cases - Cases F, G, H and I - immunohistochemical analysis demonstrated that the SCID tumours all

showed an identical monoclonal Ig phenotype to that of the original biopsies. All of the original biopsies, as expected, showed monoclonal Ig expression - IgM, $\kappa$  (cases F and G), IgG, $\lambda$  (Case H) and IgG, $\kappa$  (Case I). This Ig expression is in keeping with the pattern observed for follicle centre cell-derived NHL - although IgM is found to be the predominant isotype expressed among all histologic types of NHL, there is a high expression of IgG among follicle centre cell-derived lymphomas (Case J was also IgG, $\lambda$ ). IgG has also been reported to be the predominant isotype in immunoblastic lymphoma (Gorden 1984).

Each SCID tumour retained the Ig isotype of their original biopsy, and the IgM, $\kappa$  phenotype was retained throughout the passage of Case F SCID tumours. This is consistent with these SCID tumours being derived from the malignant clone in their original biopsies. Indeed, in cases F and G, an identical clonal IgH gene rearrangement was found in the original biopsy and the resultant SCID tumours, this clonal rearrangement being identical throughout the passages of the Case F SCID tumours demonstrating that this malignant clone is maintained successfully through passage of the SCID tumour. Therefore, this confirms the Ig expression analysis, and there is definite evidence that these SCID tumours are representative of the neoplastic clone present in the original biopsies. Unfortunately, IgH gene rearrangement analysis was not available to confirm the Ig expression results of SCID tumour H1. An IgH gene rearrangement was not detected either in Case I or SCID tumour I1 by PCR. Unfortunately Southern blot analysis could not be carried out on Case I because of DNA degradation, but a clonal IgH rearrangement was found in SCID tumour I1 by Southern blot analysis. Although a direct comparison is not possible, the fact that neither the original biopsy nor the SCID tumour showed an IgH gene rearrangement on PCR suggests that the 2 rearrangements were both in the 20% not picked



up by PCR. This is supportive of an identical rearrangement in the original biopsy and SCID tumour - unfortunately however this cannot be proven.

Ig secretion is characteristic of EBV-immortalised non-neoplastic B cells. Cells recently immortalised by EBV secrete Ig (reviewed by Nilsson and Klein 1982)) and this was also found in the EBV positive SCID tumours derived from inoculation with PBL from EBV sero-positive donors. These mice had detectable mono, oligo or polyclonal Ig in their sera (Nadal et al 1992, Purtillo et al 1992, Veronese et al 1994). These SCID tumours are obviously derived from proliferation of EBV-transformed normal B cells present in the inoculate, allowed to grow in the immuno-deficient host. Since it was suspected that our EBV (EBER/LMP/EBNA-2) positive SCID tumours - all 7 HD-derived, J1, K1, K2, K3 and K4 SCID tumours - could be derived simply from proliferation of EBV positive bystander B cells, one would expect those SCID tumours to show secretion of Ig as a feature of EBV-immortalised normal B cells, and the Ig secretion results confirmed this hypothesis. As stated before, the EBV status of the SCID tumours fell into 3 main groups:- a non-restrictive Latency Type III EBV pattern of infection (EBER/LMP/EBNA-2 positive) including all 7 of the HD-derived SCID tumours and NHL-derived SCID tumours J1, K1, K2, K3 and K4; a restricted Latency Type I EBV pattern of infection (EBER positive only) including Case L-derived SCID tumours; and EBV negative including cases F, G, H and I-derived SCID tumours. Using these groups, the Ig secretion analysis showed an interesting pattern.

Although no blood was available for SCID tumours J1 and K4, all the other EBV (EBER/LMP/EBNA-2) positive SCID tumours showed Ig secretion, thus showing features of EBV-transformed normal B cells. The Ig isotypes secreted correlated very well to the Ig isotypes expressed by the tumour cells, corresponding to in each case an EBV-transformed dominant B cell



clone secreting its immunoglobulin, the major Ig isotypes secreted being of this clone because of its predominance in the SCID tumour, with the other EBV-transformed B cell sub-clones also secreting their Ig, these being of a lower secretion because they are minor clones within the SCID tumour.

The EBER positive only SCID tumours (Case L-derived SCID tumours) however, did not secrete Ig. The characteristics of this restricted EBV pattern of infection are demonstrated here - this Latency I EBV phenotype is also seen in BL cell lines which retain the malignant phenotype of the original biopsy, they also do not secrete Ig (only under exceptional circumstances). EBV clonality analysis and IgH gene rearrangement studies are consistent with these SCID tumours being derived from the malignant clone in the original biopsy and they are not showing features of EBV-transformed normal B cells, but retaining the malignant EBV phenotype of the original LCAL biopsy. The 'mixed' EBV phenotype of SCID tumour L4 is shown very neatly here, with 'mixed' characteristics of the 2 EBV phenotypes present - the EBER positive IgA-expressing, large anaplastic cells present do not secrete their Ig, only the EBV-transformed, EBER/LMP/EBNA-2 positive IgM, $\kappa$  -expressing immunoblasts are induced to secrete their Ig. Again it is demonstrated here that the LMP/EBNA-2 positive immunoblasts present in SCID tumour L1 are in such a minority as to be considered dismissable, since no Ig secretion is seen in the SCID tumour.

Further evidence that the Ig secretion is linked to EBV is the fact that none of the EBV negative SCID tumours secreted Ig. The exception was SCID tumour I1, however this only secreted a low level of IgG - the O.D. reading of 0.075 was much lower than the O.D. results of the major isotypes secreted by the other SCID tumours. This was except for SCID tumour K3, but this SCID tumour was much smaller than I1 and would be expected to secrete less Ig because of its small size - I1 was a bigger SCID tumour and

therefore it secreted comparatively less Ig. However, I1 was derived from an immunoblastic lymphoma, which are known to secrete high levels of Ig, possibly a reflection of an intermediate cell type developing toward Ig-secreting plasma cells - plasmacytoid cells can be common in immunoblastic lymphomas (Stansfeld 1992b). Indeed, it was noted that in the I1 SCID tumour, although morphologically resembling the original biopsy very closely, the immunoblasts showed more abundant cytoplasm i.e. more plasmacytoid differentiation. Therefore, this Ig secretion in SCID tumour I1 seemed to be simply a reflection of its phenotype.

## **Chapter Seven**

### **DNA Ploidy of Original Biopsies and SCID Tumours**

Because of the involvement of the Epstein-Barr virus in a proportion of these SCID tumours, DNA ploidy was carried out to determine whether the SCID tumours were aneuploid as seen in malignant cells or had the normal diploid DNA content, seen in EBV-transformed normal B cells.

#### **7.1 DNA Ploidy of the Original Biopsies and SCID Tumours**

##### **HD Biopsies**

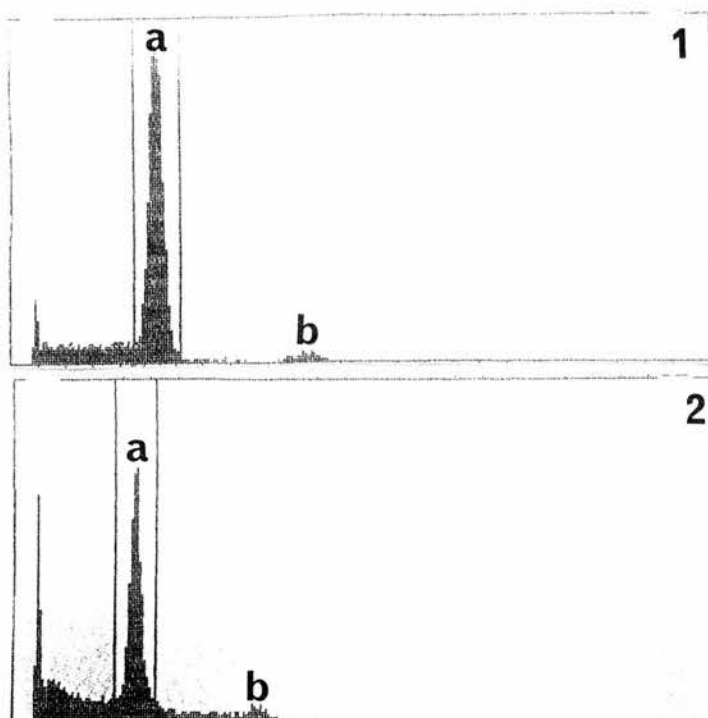
Aneuploidy was not detected in any of the 5 HD biopsies. A clear single diploid G0/G1 DNA peak was detected in each case, with a small diploid G2-M peak (G2-M cell population range was 0.2 - 4.8%). All DNA indices were 1 (Table 7.1) (Figure 7.1).

##### **HD-Derived SCID Tumours**

The results of the DNA ploidy are shown in Table 7.1. All 7 of these SCID tumours were found to contain the normal diploid DNA content - one diploid G0/G1 DNA peak and a small G2-M peak was seen. G2-M cell populations ranged from 0.4 - 12.2% and all had a DNA Index of 1 (Figure 7.1).

**Table 7.1    DNA Ploidy of Original Hodgkin's Disease Biopsies and SCID Tumours as Detected by Flow Cytometric Analysis**

CASE (HD Biopsy/SCID Tumour)	DNA PLOIDY ANALYSIS		
	G2-M%	DNA INDEX	PLOIDY
Case A - LPHD SCID Tumour A1	0.2 2.1	1 1	DIPLOID DIPLOID
Case B - LPHD SCID Tumour B1 SCID Tumour B2	0.8 5.0 0.4	1 1 1	DIPLOID DIPLOID DIPLOID
Case C - LPHD SCID Tumour C1 SCID Tumour C2	1.8 5.9 12.2	1 1 1	DIPLOID DIPLOID DIPLOID
Case D - NSHD SCID Tumour D1	2.2 3.9	1 1	DIPLOID DIPLOID
Case E - MCHD SCID Tumour E1	4.8 2.2	1 1	DIPLOID DIPLOID



**Figure 7.1 DNA Ploidy of Original Hodgkin's Disease Biopsy and SCID Tumour**

**Graph 1**      Original HD biopsy Case C LPHD showing DNA diploidy

**Graph 2**      HD-derived SCID tumour B1 showing DNA diploidy

**Key**

**a**      diploid G0/G1

**b**      diploid G2/M



### NHL Biopsies

Aneuploidy was not detected in any of the NHL biopsies (G2-M cell populations ranged from 0.6 - 11.9%) apart from Case F centroblastic lymphoma which, with a DNA Index of 2.05 and a G2-M cell population of 19%, appeared to be tetraploid (Table 7.2)(Figure 7.2).

### NHL-Derived SCID Tumours

The DNA ploidy results are given in Table 7.2. SCID tumour J1 contained the normal diploid DNA content, with a G2-M cell population of 2.0 and a DNA Index of 1.

The SCID tumours derived from Cases F, G, H and I were all aneuploid. SCID tumour I1 was aneuploid, an extra aneuploid DNA peak being present in addition to the normal diploid DNA G0/G1 and G2/M peaks. The cell population in this aneuploid G0/G1 was 81%, the DNA Index being 1.21 (Figure 7.2). A small aneuploid G2-M DNA peak was also seen.

The remaining SCID tumours derived from Cases F, G and H showed tetraploid aneuploidy with DNA indices ranging from 1.98 - 2.16, and all having more than 15% of the total cell population in G2-M (Kallioniemi et al 1988). In most cases, a small normal diploid DNA peak, a very large tetraploid G0/G1 peak (masking the diploid G2-M) and a clear tetraploid G2-M peak were seen.

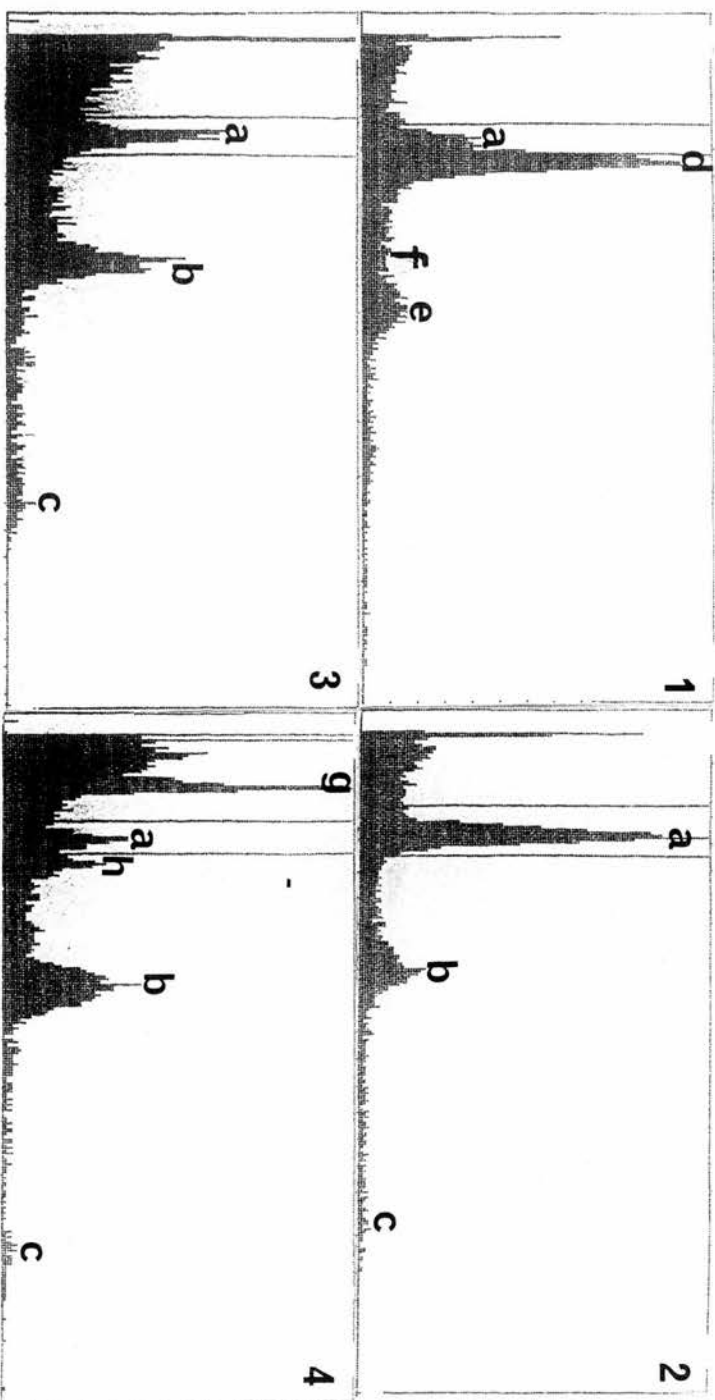
The SCID tumours derived from Case F were all tetraploid. A large tetraploid G0/G1 peak with a clear tetraploid G2-M DNA peak was seen in each case (Figure 7.2). The full list of F SCID tumour's G2-M cell populations (entered in Table 7.2 as a mean result) are as follows :- F1=55.1%, F2=46.1%, F3=55.3%, F4=58.1%, F5=46.1%, F6=50.1%, F7=51.7%, F8=54.9%, F9=48.2%, F10=38.9%, F11=69.2%, F12=63.9%, F13=47.2%. DNA ploidy analysis was not carried out on F14.

**Table 7.2    DNA Ploidy of the Original Non-Hodgkin's Lymphoma Biopsies and SCID Tumours as Detected by Flow Cytometric Analysis**

CASE (NHL Biopsy/SCID Tumour)	DNA PLOIDY ANALYSIS		
	G2-M%	DNA INDEX	PLOIDY
Case F - NHL Cb SCID Tumours F1 - F14 SCID Tumour F5	19.0 Mean - 52.6 46.1	2.05 Mean - 2.08 0.59/2.05	TETRAPLOID ALL TETRAPLOID HYPODIPLOID/ TETRAPLOID
Case G - NHL Cb SCID Tumour G1	2.4 28.3	1 1.99	DIPLOID TETRAPLOID
Case H - NHL Cb SCID Tumour H1	0.6 16.0	1 1.98	DIPLOID TETRAPLOID
Case I - NHL ImmB SCID Tumour I1	2.2 #81	1 1.21	DIPLOID ANEUPLOID
Case J - NHL Cb/Cc Foll SCID Tumour J1	5.5 2.0	1 1	DIPLOID DIPLOID
Case K - LCAL SCID Tumour K1 SCID Tumour K2 SCID Tumour K3 SCID Tumour K4	11.9 3.3 6.3 NM 8.3	1 1 1 - 1	DIPLOID DIPLOID DIPLOID - DIPLOID
Case L - LCAL SCID Tumour L1 SCID Tumour L2 SCID Tumour L3 SCID Tumour L4 SCID Tumour L5	3.0 19.8 78.2 84.4 80.2 78.4	1 2.03 2.11 2.15 2.16 2.16	DIPLOID TETRAPLOID TETRAPLOID TETRAPLOID TETRAPLOID TETRAPLOID

**key**

NM    No Material  
#    Refers to cell population present in the near diploid DNA peak



**Figure 7.2 DNA Ploidy of Original Non-Hodgkin's Lymphoma Biopsy and SCID Tumours**

**Graph 1** NHL-derived SCID tumour I1 showing near-diploid aneuploidy

**Graph 2** NHL biopsy Case F - the only original biopsy to show tetraploidy

**Graph 3** Case F-derived SCID tumour F2 showing tetraploidy

**Graph 4** Case F-derived SCID tumour F5 was unusual in that it showed a hypodiploid and a tetraploid DNA peak

**Key**

- |   |                                   |   |                              |   |                   |
|---|-----------------------------------|---|------------------------------|---|-------------------|
| a | diploid G0/G1                     | d | near-diploid aneuploid G0/G1 | g | hypodiploid G0/G1 |
| b | diploid G2-M and tetraploid G0/G1 | e | aneuploid G2-M               | h | hypodiploid G2-M  |
| c | tetraploid G2-M                   | f | diploid G2-M                 |   |                   |

SCID tumour F5 was unusual in that it showed both hypodiploid (DNA Index of 0.59) and tetraploid (DNA Index of 2.05) cell populations. A large hypodiploid G0/G1 DNA peak, a normal diploid G0/G1 DNA peak, a hypodiploid G2-M DNA peak, a large tetraploid G0/G1 DNA peak (masking the diploid G2-M DNA peak) and a tetraploid G2-M DNA peak were all seen (Figure 7.2).

SCID tumour G1 was also tetraploid with a G2-M cell population of 28.3% and a DNA Index of 1.99, as was SCID tumour H1 with a G2-M cell population of 16% and a DNA Index of 1.98.

The 2 sets of SCID tumours derived from the LCAL (Cases K and L) again produced different results. The 4 SCID tumours derived from Case K all appeared to contain the normal diploid DNA content, each displaying a single diploid G0/G1 DNA peak with a small G2-M DNA peak (Figure 7.3). The G2-M cell populations ranged from 3.3 - 8.3, all having a DNA Index of 1 (SCID tumour K3 could not be analysed for DNA ploidy because of the insufficient material available).

In contrast, the 5 SCID tumours derived from Case L (L1 - L5) all displayed tetraploid aneuploidy. SCID tumour L1 contained the lowest tetraploid peak, but the remaining 4 each showed a huge tetraploid peak, and a clear tetraploid G2-M peak was seen in each case (Figure 7.3).

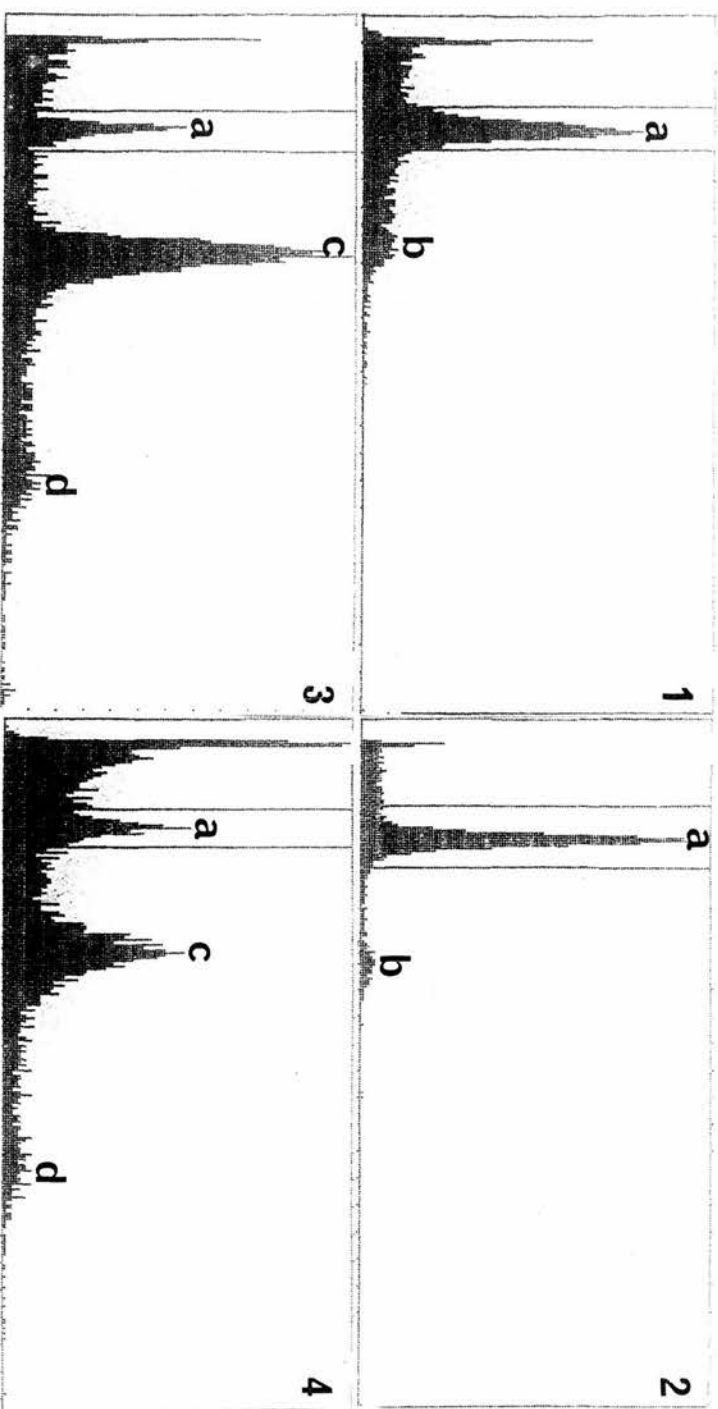
As can be seen from Table 7.3, all of the EBV (EBER/LMP/EBNA-2) positive SCID tumours were found to contain the normal diploid DNA content (SCID tumours A1, B1, B2, C1, C2, D1, E1, J1 and K1-4). In contrast, the EBER positive only SCID tumours (even those with sub-populations of LMP/EBNA-2 positive cells - L1 & L4) and the EBV negative SCID tumours - F1-14, G1, H1 and I1 were all aneuploid.

**Table 7.3**

**DNA Ploidy of SCID Tumours - Correlation  
With EBV Status**

SCID TUMOUR	EBV STATUS	PLOIDY
A1	POS	DIPLOID
B1 B2	POS POS	DIPLOID DIPLOID
C1 C2	POS POS	DIPLOID DIPLOID
D1	POS	DIPLOID
E1	POS	DIPLOID
F1 - F14 F5	ALL NEG NEG	ALL TETRAPLOID HYPODIPLOID/TETRAPLOID
G1	NEG	TETRAPLOID
H1	NEG	TETRAPLOID
I1	NEG	ANEUPLOID
J1	POS	DIPLOID
K1 K2 K3 K4	POS POS POS POS	DIPLOID DIPLOID - DIPLOID
L1 L2 L3 L4 L5	EBER(LMP/EBNA-2) EBER EBER EBER(LMP/EBNA-2) EBER	TETRAPLOID TETRAPLOID TETRAPLOID TETRAPLOID TETRAPLOID





**Figure 7.3 DNA Ploidy of original Large Cell Anaplastic Lymphoma Biopsy and SCID Tumours**

- Graph 1 LCAL-derived (Case K) SCID tumour K2 showing DNA diploidy  
 Graph 2 Original LCAL biopsy Case L showing DNA diploidy  
 Graph 3 Case L-derived SCID tumour L2 showing tetraploidy  
 Graph 4 Case L-derived SCID tumour L4 showing tetraploidy

Key			
a	diploid G0/G1	c	tetraploid G0/G1 + diploid G2-M
b	diploid G2/M	d	tetraploid G2/M

## **7.2 Comment**

Aneuploidy was not detected in any of the original HD biopsies in this study. This is perhaps not surprising because of the scarcity of the neoplastic RS/H cells amongst the normal diploid reactive cellular infiltrate. Aneuploidy has been detected in HD (Morgen et al 1988), being more common in the NS, MC and LD sub-types, possibly because of the higher numbers of RS/H cells present in these sub-types. Over-all however, only 11% (13/115 cases) showed aneuploidy in the Morgen et al study. This was also found by Joensuu et al (1988) who detected DNA aneuploidy by flow cytometric analysis in 11% (8/70) of their HD cases. In a study by Anastasi et al (1987), DNA aneuploidy was only found in 1 of the 15 cases of HD studied by flow cytometric DNA analysis alone, but evidence of DNA aneuploidy was considered to be present in all 15 cases if a combined analysis of DNA content and nucleolar antigens was performed. This technique was also used by Erdkamp et al (1994), who detected DNA aneuploidy in 49% (67/137) of HD cases studied.

Aneuploidy was detected in only one of the original NHL biopsies in this study - Case F NHL Cb (tetraploid).

Karyotyping has been found to show chromosomal abnormalities in between 75-95% of NHL cases. The most common abnormality in NHL appears to be near-diploid chromosomal numbers i.e. 44-49. It has been estimated that an increase or decrease in nuclear DNA has to be 5% before detectable by flow cytometric analysis, therefore near-diploid aneuploidy may not be picked up (Woolridge et al 1988). In another study, only 19% of NHL cases were found to be aneuploid by flow cytometry (cytogenetic analysis showed that 92% of these cases had chromosomal abnormalities), however all specimens shown to have a chromosome number exceeding 50 by karyotypic analysis were DNA aneuploid by flow cytometric analysis

(Lakkala et al 1990). Therefore, it appears that flow cytometry may be a rather coarse method, only detecting aneuploidy when there is a major increase in chromosome number.

Therefore, it is possible that the original NHL biopsies showed a near-diploid aneuploidy which was not picked up by the flow cytometer. However, this would seem especially unlikely in the LCAL biopsies (Cases K and L), a high chromosome number being characteristic of large cell lymphomas (Levine et al 1985). However, in a study of DNA ploidy analysis of LCAL cases using the flow cytometer, aneuploidy was not detected in any case and there was found to be no association between tumour histologic characteristics and detection of DNA aneuploidy (Lakkala et al 1990).

Therefore, it is possible that near-diploid and even tetraploid aneuploidy was present in the original NHL biopsies in this study and not picked up by the flow cytometric analysis.

Normal non-neoplastic B cells, infected by EBV, retain their normal diploid DNA content. Chromosomal analysis has been carried out on hundreds of recently established LCLs, and without exception they had a normal diploid karyotype (Nilsson and Klein 1982). Therefore, it can be concluded that the EBV infection of a B cell, although conferring to the cell one neoplastic feature (immortality), is not followed by any chromosomal alteration whatsoever. The EBV positive SCID lymphomas derived from inoculation of PBL from EBV sero-positive donors, were also all found to contain the normal diploid DNA content (Cannon et al 1990, Nakamine et al 1991, Rowe et al 1991, Mosier et al 1992, Nadal et al 1992, Purtillo et al 1992, Veronese et al 1994). These SCID tumours are obviously derived from proliferation of EBV-transformed normal B cells present in the inoculate allowed to grow in the immune-deficient host, and it was suspected that our EBV positive (EBER/LMP/EBNA-2) SCID tumours may

simply be derived from EBV-transformed bystander B cells, and the DNA ploidy results confirmed this hypothesis. The results showed that DNA ploidy can be linked very well to the EBV status of the SCID tumours, by grouping the SCID tumours into the 3 EBV status phenotypes - Latency III EBV (EBER/LMP/EBNA-2) positive, Latency I EBER only positive and EBV negative - a characteristic pattern emerged.

All 7 of the HD-derived and Cases J and K-derived SCID tumours contained the normal diploid DNA content. This is in keeping with their non-restrictive Latency III EBV phenotype, showing characteristics of EBV-transformed normal B cells, indicative of their non-neoplastic origin. It could be argued that near-diploid aneuploidy could have been missed by the flow cytometric analysis, but this is unlikely owing to their EBV status and also, 2 cases that were analysed cytogenetically showed that a normal diploid 46 XY karyotype was present (data not shown).

In contrast, the restricted EBV phenotype (EBER positive only) SCID tumours - L1-L5 - all showed tetraploid aneuploidy. A diploid DNA peak was also seen in each case - this is probably existing diploid tumour cells, it is unlikely to be normal reactive cells as no residual T cells or macrophages were detected in any of the SCID tumours. The characteristics of this restricted Latency I EBV pattern of infection are being displayed here. This phenotype is also seen in BL cell lines which retain the malignant phenotype of the original biopsy and are always aneuploid. These SCID tumours, shown to be derived from the malignant clone in the original LCAL biopsy are all showing aneuploidy. Again, these results confirm that the sub-population of LMP/EBNA-2 positive immunoblastic cells present in SCID tumour L1 are dismissable as this was found to be tetraploid. Even the SCID tumour L4, with a more prominent population of LMP/EBNA-2 positive cells was found to be tetraploid - this result confirms the morphological and

phenotypic results but not the genotypic analysis. All 5 of the Case L-derived SCID tumours displayed tetraploid aneuploidy even though this was not detected in the original biopsy. There are 2 possible explanations for this:- perhaps the original LCAL biopsy did display a near-diploid aneuploidy (not picked up by the flow cytometer) and the SCID environment (length of time growing in the SCID mouse and the lack of immune intervention) has allowed tumour progression - it has been speculated that doubling of the DNA content (i.e. tetraploidy) would be the first event in the development of DNA aneuploidy (Mauro et al 1986) However, this may seem unlikely because of the morphological appearance of the LCAL, the original Case L biopsy could contain a small tetraploid population, this not being picked up by the flow cytometer, but the SCID environment could have allowed a growth advantage to this small tetraploid malignant clone. With the purer population of tumour cells present in the SCID tumours, this tetraploidy can now be picked up by the flow cytometer. It is interesting to note that of the 2 LCAL biopsies, Case K contained the higher G2-M population, indeed the highest of all the 'diploid' original biopsies (11.9%). This may indicate that the tetraploid population in Case K (although too small to be picked up by the flow cytometer) is more evident than in Case L and it is unusual that Case K produced 4 non-related EBV-induced lymphoproliferations, whilst the malignant clone present in Case L grew in the resultant SCID tumours. Therefore, perhaps the size of the malignant population present is not important for the growth of lymphomas in SCID mice, and chance plays a part in whether EBV-induced lymphoproliferations will dominate in these SCID tumours.

All of the EBV negative SCID tumours (F1-14, G1, H1, I1) displayed aneuploidy. Therefore they are not displaying the same characteristics as that of the EBV (EBER/LMP/EBNA-2) positive SCID tumours. This is again



consistent with these SCID tumours being derived from a malignant clone in the original biopsies as shown by the genotypic analysis.

Case F original biopsy displayed tetraploid aneuploidy and this karyotype was retained throughout the SCID tumours. SCID tumour F5 was interesting in that it displayed a hypodiploid and a tetraploid tumour cell population. This may represent further tumour progression allowed by the SCID environment - it is thought that an increase or decrease (as is here) of chromosomal material in tumour cells with a tetraploid DNA content appears to be related to a more pronounced anaplasia and enhanced malignant potential (Kallioniemi et al 1988). It is unfortunate that this F5 SCID tumour was not the one used for further passage.

SCID tumours G1, H1 and I1 were all aneuploid with G1 and H1 again being tetraploid, and I1 displaying hyperdiploid aneuploidy (DI of 1.21). None of the original biopsies displayed aneuploidy and again the explanation for this difference may be as that for SCID tumours L1-L5, the SCID environment allowing a growth advantage of a small aneuploid population present in the original biopsies. This theory is demonstrated by the fact that the original Case F biopsy contained only a small tetraploid peak, whereas the SCID tumours derived from this all showed much larger tetraploid populations. The SCID environment allowing tumour progression may be a feasible explanation and may explain the prevalence of tetraploid aneuploidy amongst the SCID tumours - it was noted that morphologically, although the EBV negative NHL-derived SCID tumours resembled the original biopsies very closely, they did appear to show more nuclear pleomorphism.

Therefore, the DNA ploidy results have demonstrated the characteristics of the 3 different lymphoproliferations. It was shown that the EBV(EBER/LMP/EBNA-2) positive SCID tumours contained the normal

diploid DNA content, demonstrating that all of the HD-derived and Case J and K-derived SCID tumours share characteristics of EBV-transformed normal B cells and confirmed the hypothesis that they may be derived simply from proliferation of EBV-transformed normal bystander B cells present in the injected node. In contrast, the EBER positive only and the EBV negative SCID tumours were all found to be aneuploid, confirming the genotypic analysis that they are derived from the malignant clone in the original biopsies.

However, flow cytometric analysis of DNA ploidy can be a rather coarse method of detection of aneuploidy. Obviously, cytogenetic analysis would resolve which DNA aneuploidy was exhibited by the original NHL biopsies, if any. However, this is a time-consuming specialist technique, and was only available for use with this study during the later stage. A viable cell suspension is required for this technique, which was limiting especially from early biopsies. This may still not resolve the karyotype of the HD biopsies, as the neoplastic RS/H cells are so scarce and would be diluted by the normal diploid reactive cellular infiltrate. However, this can be resolved using the Highly Optimised Microscope Environment (HOME) - a computerised microscope which, using an integrated image analysis system, can be used to measure the DNA ploidy of individual cells. After an initial set-up, ploidy measurements can be made quickly by pointing with the mouse to the nucleus to be measured and pressing the mouse button. This would allow the DNA ploidy of the individual RS/H cells to be measured, not possible using flow cytometry (Gray et al 1994, Salto-Tellez et al 1994). Indeed, initial results have shown that the RS/H cells do exhibit aneuploidy (Krajewski - unpublished results). Unfortunately, this system was not available until after this study was complete.

# **Chapter Eight**

## **General Discussion**

Although many phenotypic and genotypic studies have been carried out, the histogenesis of the RS cell remains unknown. In order to investigate the histogenesis of the RS cell and Hodgkin's disease, this thesis attempted to establish a SCID mouse model to study the disease, and to investigate whether non-Hodgkin's lymphomas could successfully be transplanted into SCID mice.

This thesis has shown that transplantation of both Hodgkin's disease and non-Hodgkin's lymphomas into SCID mice results in the growth of high grade human B cell lymphomas. It was established that the Epstein-Barr virus had a role in a proportion of these SCID tumours. 3 main types of lymphoproliferations were obtained - EBV (EBER, LMP, EBNA-2) positive SCID tumours expressing a non-restricted EBV latency III phenotype, EBER positive only SCID tumours expressing a restricted EBV latency I phenotype and EBV negative SCID tumours. The EBV positive SCID tumours arose from both EBV positive and EBV negative original biopsies. These SCID tumours were thoroughly characterised to determine whether or not they were derived from the malignant clone in the original biopsy and thus whether they represented a good *in vivo* model for Hodgkin's disease or non-Hodgkin's lymphomas.

## **The SCID Mouse Model of Hodgkin's Disease - Overview and Conclusions**

All 7 of the HD-derived SCID tumours displayed a non-restricted latency III EBV pattern of expression - all EBER, LMP and EBNA-2 positive. This is not the EBV latency pattern seen in RS/H cells in HD, indeed only one of the original HD biopsies in this study contained EBV positive RS/H cells (Case E) and terminal repeat analysis demonstrated that this EBV positive clone of the original biopsy was not present in the SCID tumour. On this evidence alone, it would appear unlikely that these SCID tumours are derived from the neoplastic RS/H clone of the original biopsies. It is well documented that inoculation of SCID mice with PBL from EBV sero-positive donors will produce B cell lymphoproliferations in most cases (Cannon et al 1990, Nakamine et al 1991, Rowe et al 1991, Mosier et al 1992, Nadal et al 1992, Purtillo et al 1992, Veronese et al 1994). Therefore, it was a possibility that these SCID tumours were simply derived from out-growth of EBV-transformed bystander B cells present in the injected node, allowed to proliferate in the immuno-deficient host, and extensive characterisation was carried out to test this hypothesis.

Morphologically, the HD-derived SCID tumours resembled LCL, the SCID tumours following injection of normal EBV positive PBL and the EBV positive lymphomas seen in immunocompromised individuals, not RS/H cells and the histological appearance of HD. Phenotypically, they possessed an activated B cell phenotype with high expression of the EBV-associated antigens CD23 and CD43 and furthermore displayed characteristics of EBV-immortalised normal B cells in their diploid DNA content and Ig secretion. Therefore, it was demonstrated that these SCID tumours derived from injection of fresh HD biopsy material shared all the characteristics of EBV-

immortalised normal B cells and were most probably simply derived from out-growth of EBV-transformed bystander B cells present in the injected lymph node/spleen suspension, and this SCID mouse animal model is not a satisfactory *in vivo* model for Hodgkin's disease. These SCID tumours did express a predominant single immunoglobulin heavy and light chain and showed a clonal IgH gene rearrangement by PCR, but monoclonal EBV positive SCID tumours have been found in the PBL-derived SCID tumours and in LCL (Rowe et al 1991, Nakamine et al 1993) and considering the small number of EBV positive bystander B cells injected within the HD lymph node, it is perhaps not surprising that only one dominant clone of EBV positive B cells was seen.

During this thesis, another group carried out similar work. Kapp et al (1993) grew human SCID tumours from 3/13 cases of fresh HD biopsy material, a slightly lower success rate than this thesis, but in contrast to this study, 100% tumour take was achieved in 2 cases (6/6 and 3/3) - perhaps the mode of injection used in the Kapp study (sub-renal and intra-hepatic implantation) was more suitable, although additional problems such as inadequate tumour tissue for passage and 'passage' mice being killed by infection were encountered in this thesis. The ip route of injection used in this thesis also does not allow a proper study of metastasis, however the one subcutaneous SCID tumour grown (E1) appeared to remain localised to the site of injection, no spread to other tissues was seen.

In common with this thesis, all of the HD-derived SCID tumours obtained in the Kapp study expressed EBER, LMP and EBNA-2, even though 2 of the original HD biopsies were EBV negative. Kapp et al described 3 distinct histological lesions in their SCID tumours - (i) lymphoproliferative disease - lesions consisting of medium-sized B cells, B-immunoblastic-like cells and cells with plasmacytoid features (ii) large cell



anaplastic lymphomas (iii) HD-like lesions showing RS-like cells surrounded by murine macrophages. The lymphoproliferative disease described by the Kapp study resembles the polymorphous immunoblastic proliferations seen in this thesis. None of our HD-derived SCID tumours showed lesions with definite features of LCAL or were HD-like, but SCID tumours C1 and C2 showed some features of LCAL and areas of increased cellular polymorphism could be seen in most cases. Occasional bi-nucleate or multi-nucleated RS-like cells were also found in each HD-derived SCID tumour. Kapp et al hypothesised that because of the morphology of these LCAL lesions and the RS/H-like cells in their so-called HD-like lesions, they were likely to be derived from the RS/H cells in the original biopsies. However, these cells, like the cells in their LPD lesions displayed a non-restricted latency III EBV pattern of infection (EBER/LMP/EBNA-2 positive), not the latency II EBV pattern of infection (EBER/LMP positive) seen in RS/H cells, only one of the original biopsies being EBV positive in this Kapp study. Also, RS/H-like cells have been found in the benign condition infectious mononucleosis (Isaacson et al 1992), therefore perhaps this morphology was EBV-induced, as with the high CD30 expression seen, not only seen in their RS/H-like or LCAL-like cells but in 50-80% of the SCID tumour cell population. Kapp et al did not carry out EBV clonality analysis to determine whether the EBV positive clone in the only EBV positive original biopsy was also present in the corresponding SCID tumour.

Kapp et al reported that their HD-derived SCID tumours showed a high incidence of chromosomal abnormality, evidence that they were derived from the malignant RS/H cells. However, of the karyotypes they reported, some were diploid and others showed a near diploid chromosome number. It is possible that near-diploid aneuploidy was missed in this thesis by the flow cytometry analysis - abnormal mitoses could be seen in each SCID

tumour, however 2 cases analysed cytogenetically showed a normal 46XY karyotype (data not shown). Also, clonal chromosomal changes do occur in the lymphomas arising in SCID mice inoculated with PBLs from normal EBV sero-positive donors (Thangavelu et al 1992, Glaser et al 1993). Therefore, it is possible that the chromosomal abnormalities detected by Kapp et al may have arisen in normal cells spontaneously following transplantation into the mice.

In this thesis, expression of the LMP and EBNA-2 was seen in only a proportion of the tumour cells in the EBV (EBER/LMP/EBNA-2) positive SCID tumours (LMP - 30-40%, EBNA-2 - 10-20%). It has been noted before that the EBV-transformed B cells *in vivo* (i.e. the EBV positive PBL-derived SCID lymphomas) showed reduced EBV latent gene expression (LMP/EBNA-2) compared to EBV-transformed B cells *in vitro* (LCL) (Rochford & Mosier 1995). This is also shown when LCL are transplanted into SCID mice (Rochford et al 1993). It is thought that this reduction in EBV latent gene expression correlates with B cell differentiation *in vivo* as the SCID tumour cells showed a plasmacytoid differentiation compared to the lymphoblastoid phenotype of the *in vitro* LCL. This was also linked with lower CD23 expression and higher Ig secretion *in vivo* (Rochford et al 1993).

This could explain the lower latent gene expression seen in the EBV(EBER/LMP/EBNA-2) positive SCID tumours in this thesis as they did show plasmacytoid differentiation and high Ig secretion, however they also showed high CD23 expression. It is also possible that EBV has entered the lytic cycle - recently Rochford & Mosier (1995) found correlation of lytic viral gene expression with a more differentiated B cell phenotype (plasmacytoid differentiation, high Ig secretion, low CD23 expression) in the EBV sero-positive donor PBL-derived SCID tumours. Rochford & Mosier (1995) have proposed that the SCID environment allows proliferation of latently-infected

B lymphoblasts which then differentiate to plasmacytoid cells with reactivation of lytic cycle infection and release of new virus which infects bystander B cells, some of which then proliferate as latently-infected lymphoblasts - since most of these SCID tumours are oligoclonal, some monoclonal, the rate of generation of secondary transformants must be low (Rochford & Mosier 1995).

Lytic viral gene expression was not looked for in the EBV (EBER/LMP/EBNA-2) positive SCID tumours in this thesis as it was not central to the main objective of whether or not these EBV positive SCID tumours were derived from the original HD or NHL clone. However, it is feasible, and this could explain the lower latent gene expression in these SCID tumours.

Also, it is not beyond all possibility that the HD-derived SCID tumours obtained in this thesis did contain some RS/H cells grown from the original biopsy. RS-like cells were seen in each HD-derived SCID tumour, but not in the other EBV (EBER/LMP/EBNA-2) positive SCID tumours (J1, K1, K2, K3, K4). It is possible that these RS cells were infected by EBV virus released from the surrounding B cells in the SCID tumours.

However, the Latency II type of infection seen in the larger blasts and the RS/H-like cells in the HD-derived SCID tumours (LMP+/EBNA-2 negative) cannot be taken as representing the Latency II pattern of expression seen in RS cells of Hodgkin's disease, as Oudejans et al (1995) recently found in post-transplant lymphomas, using combined EBER *in situ* hybridisation and immunocytochemistry (including double-staining) to investigate EBV expression at a single cell level, that cells expressing both EBNA-2 and LMP were rarely detected. Although an overall Latency III pattern was expressed, a mixture of at least 3 different cell populations were identified - (I) EBER+/EBNA-1+, (II) EBER+/EBNA-1+/LMP+ and

(III) EBER+/EBNA-1+/EBNA-2+ - ie a population of LMP+/EBNA-2 negative cells were present here also. Therefore, the pattern of expression seen in the HD-derived SCID tumours has also been found in post-transplant lymphomas and may not be a consequence of the injection of RS cells or their precursors. If the LMP+/EBNA-2 negative RS-like cells in the SCID tumours were derived from injected RS cells, obviously these cells are the absolute minority, completely out-numbered by the EBV positive lymphoproliferation and as such would not represent an *in vivo* model for HD. There can be no way to prove this however, and if further studies were to be carried out, they would require cases of HD with clonotypic markers such as clonal EBV, clonal Ig gene rearrangements or cytogenetic markers to allow comparison with the SCID tumours. Obviously, it would be more useful to include more cases of NS, MC and LD sub-types of HD since LPHD is now widely accepted to be of B cell origin.

Recently, Shpitz et al (1994) established a SCID mouse model which allowed improved engraftment of human lymphoid cells and tumours by pre-treating the SCID mice with sub-lethal radiation and an anti-asialo-GM1 antibody to eliminate NK cell activity. However, it is possible that the SCID mouse would never be a suitable model for HD - the histological picture of HD is the scarce neoplastic RS/H cells amongst a significant reactive lymphoid cellular infiltrate including B and T cells, eosinophils, neutrophils and macrophages. Perhaps the growth of HD requires an intact, functioning immune system with cell-cell and cytokine interactions between the RS/H cells and the cellular infiltrate. Many cytokines have been detected in the RS/H cells (Hsu et al 1986, Kretschmer et al 1990, Samoszuk & Nansen 1990, Jucker et al 1991, Merz et al 1991, Xerri et al 1992, Hsu et al 1993). Expression of IL-6 and the IL-6 receptor have both been found in RS/H cells (Jucker et al 1991) and it was suggested that this IL-6, either by an autocrine

or paracrine (produced by back-ground CD4 positive T cells) loop may stimulate the growth of the neoplastic RS/H cells. It would be ideal to inject IL-6 into the SCID mice along with the HD biopsy material in the hope of growth stimulation of the RS/H cells, however the problem being that this would also stimulate growth of the EBV-transformed B cells (Nadal et al 1992).

It would be difficult to eliminate the EBV positive bystander B cells present in the lymph node/spleen cell suspension prior to injection into the SCID mice not only because of their small number, but because of the risk of eliminating crucial cells such as RS/H cells or their precursors. Maybe the injection of an anti-LMP antibody into the SCID mice would be a solution for the growth of EBV negative HD cases. Alternatively, administration of the anti-viral agent ganciclovir may inhibit the development of the EBV-induced lymphoproliferations - this was shown to inhibit the EBV positive SCID lymphomas derived from injection of PBL from EBV sero-positive donors (Boyle et al 1992).

Therefore, the various phenotypic and genotypic studies carried out on HD have allowed a detailed description of RS/H cell characteristics. They express activation antigens, in some cases they express B cell-associated and in others T cell-associated antigens. Genotypic analysis has shown that they can exhibit Ig or TcR gene rearrangements, both or neither. They exhibit chromosomal abnormalities but no unique and defining aberration and can express a number of cytokines but again no unique pattern is found. The Epstein-Barr virus is known to be associated in around 40-60% of cases - however, this does not imply that the RS/H cell is of B cell origin as EBV has been found in T cell lymphomas (Weiss et al 1987, Staal et al 1989, Hamilton-Dutoit et al 1992, Sabourin et al 1993) and to infect epithelial cells in NPC (reviewed by Miller 1990). Therefore, these features



do not offer a definitive nature or origin for the RS/H cell, at most they describe a lymphoid cell.

The heterogeneous nature of HD suggests that it may be a syndrome encompassing closely related but biologically distinct entities rather than a single disease. As described earlier, the nodular LP sub-type of HD is clearly a B cell variant of the disease. Recently, it was found that in cases of LPHD with concurrent or subsequent B cell Large Cell Lymphoma, there was a clonal relationship between the 2 diseases with the authors hypothesising that the Large Cell Lymphoma represents a clonal progression of LPHD, being part of a spectrum of B cell malignancy, with polyclonal Progressive Transformation of Germinal Centres progressing to oligoclonal LPHD to monoclonal Large Cell Lymphoma (Wickert et al 1995). It may well be that a T cell variant of Hodgkin's disease also exists. When studying comparisons between HD and the T cell lymphomas lymphomatoid papulosis, mycosis fungoides and the Ki-1 positive LCAL (usually a T cell malignancy), this has prompted some authors to suggest that these disorders are related and represent a spectrum of T cell malignancy (Kadin 1985, Orscheschek et al 1995).

It has also been suggested that HD is a heterogeneous group of disorders in which viral transformation of an immature lymphoid cell plays a major role in the induction of the aberrant lymphoid cell phenotype of the RS/H cells (Herbst et al 1989, Stein et al 1991). Khan & Coates (1994) have recently hypothesised that LMP expression may interfere with the differentiation pathway of the RS cell, as it is known to inhibit the differentiation of epithelial cells, thought to be important in the pathogenesis of Nasopharyngeal Carcinoma. In Hodgkin's disease, undifferentiated cells may show an activated phenotype also as a consequence of EBV infection and it may correlate with the peculiar morphology of the RS/H cells.

Therefore, LMP expression could account for 2 of the main features of Hodgkin's disease, namely the undifferentiated phenotype and the characteristic morphology of the malignant cell population. Obviously this could only apply to those cases which were EBV positive.

It has also recently been suggested that there may be a relationship between CD30 antigen expression by RS/H cells and the pathogenesis of Hodgkin's disease (Haluska et al 1994) based on the knowledge that CD30 has been identified as a signal-transducing molecule in a specific sub-set of CD45Ro+ T cells (Ellis et al 1993). CD30 has been shown to be preferentially expressed by CD4+ T cells producing Type II cytokines (ie TH2) such as IL-4 and IL-5 (Del Prete et al 1995), and Pizzolo et al (1994) suggest that CD30 expression reflects a TH2-type functional attitude of RS/H cells in Hodgkin's disease thus explaining the cytokine profile seen in Hodgkin's disease. This pathogenic association is backed up by the fact that a large series of Hodgkin's patients had high serum CD30 levels correlating with stage of disease and symptoms present such that higher CD30 levels were associated with a worse prognosis (Nadali et al 1994).

Recent work has shown that the CD40 ligand (CD40L) is strongly expressed on the reactive CD4+ T cells surrounding RS cells. This suggests that in Hodgkin's disease tissues, CD40L+/CD4+ T lymphocytes might interact with the tumour cells via CD40 - this may result in the transmission of growth signals to RS cells (Carbone et al 1995). Recent work has also demonstrated that both the CD30 ligand and CD40 ligand present on bystander cells cause enhanced cytokine secretion from CD30+ and/or CD40+ RS/H cells (Gruss et al 1995). Therefore, the interaction of both CD30 and CD40 on RS/H cells with their ligands present in the background infiltrate may contribute to the pathogenesis and deregulated cytokine network present in Hodgkin's disease.

It may also be that the specific pattern of cytokine secretion from the reactive infiltrate present may determine the differentiation pathway of the RS/H cell. Trumper et al (1993) recently used a single cell based PCR analysis system which allows general amplification of cDNA from single cells and studied the gene expression pattern of single RS/H cells from cases of HD. They found that single RS/H cells co-express genes characteristic of several haemopoietic lineages, including macrophage, B and T cell. There was considerable case to case heterogeneity of gene expression, even in cases showing the same histology, with consistent expression of genes within cases. A group of genes was expressed by the large majority of RS cells examined; this included *c-myc*, the *c-fes/fps* oncogene, the *fyn* tyrosine kinase, the IL-2 receptor beta chain, TNF- $\beta$  and CD4. It appears from this that RS/H cells represent activated haemopoietic cells capable of expressing genes from several haemopoietic lineages. Differentiation of the tumour cells from individual patients may occur along monocytoid or lymphoid pathways, influenced by the micro environment e.g. cytokine release from the surrounding reactive cells in different cases (Trumper et al 1993).

This type of analysis i.e. looking at single cells, appears to be the most appropriate in the case of Hodgkin's disease where the scarcity of the neoplastic RS/H cells, among the normal reactive cellular infiltrate, has been the problem in the previous immunophenotypic and genotypic studies and also in this thesis. Indeed, several groups have recently employed this methodology to look at TcR and Ig gene rearrangements in Hodgkin's disease. However, conflicting results have again arisen. Roth et al (1994) did not find rearrangement of the IgH gene locus in isolated RS/H cells from 13 cases of Hodgkin's disease. Daus et al (1995) did not find rearrangement of the TcR  $\gamma$  gene in isolated RS/H cells in 13 cases of Hodgkin's.

Delabie et al (1994), looking at single RS/H cells from 4 cases of LPHD, found only polyclonal populations (they suggest that LPHD is polyclonal at an early stage, but as the L & H cells slowly continue to proliferate, they become progressively more oligoclonal and progress to monoclonal Large Cell Lymphomas - as discussed previously - page 190). However, Kuppers et al (1994), looking at single RS/H cells from 3 cases of Hodgkin's disease, found clonal IgH gene rearrangements in all 3 cases. In the MCHD case in this study, sequence analysis revealed only non-functional V gene rearrangements, suggesting a pre-B cell origin whilst in the LPHD case, there was evidence of ongoing somatic mutation of the rearranged V region genes within the tumour suggestive of a germinal centre origin. They concluded that this indicates that RS/H cells can originate from B cells at various stages of development.

At variance with these previous studies, Hummel et al (1995) found by analysing single RS/H cells from 12 cases for IgH gene rearrangements, 3 cases contained a monoclonal population of RS/H cells, 6 cases polyclonal and the remaining 3 a mixture of both polyclonal and monoclonal RS/H cells.

They suggest that technical factors may have contributed to the failure of Roth et al (1994) to detect any IgH gene rearrangements in their samples, and blame small case numbers on the discrepancy between their results and those of Delabie et al (1994) and Kuppers et al (1994).

They suggest that polyclonal populations of RS cells arise from the continuous recruitment of unrelated B lymphocytes, transformed into RS/H cells if susceptible to genetic instability, by transforming agents such as EBV, or an immune defect that impairs the elimination of aberrant cells.

Therefore, although conflicting results have arisen using this single cell technique, only a limited number of cases have been investigated as yet and it is likely that future progress will depend on this kind of methodology.



## **The SCID Mouse Model of Non-Hodgkin's Lymphomas - Overview and Conclusions**

The transplantation of NHL into SCID mice was more successful in this thesis. However, 2 cases (Cases J and K) again gave rise to EBV (EBER/LMP/EBNA-2) positive SCID tumours, which did not share the phenotypic and genotypic characteristics of the original biopsies, instead resembling EBV-transformed normal B cells. Like the HD-derived SCID tumours, these were most probably derived from proliferation of EBV positive bystander B cells present in the injected lymph node and detailed analysis of the SCID tumours demonstrated that they shared the characteristics of the HD-derived SCID tumours (the activated B cell phenotype with high expression of EBV-associated antigens, Ig secretion and normal diploid DNA content). It is interesting that one of these cases was the only low grade NHL to yield tumours in this study (Case J NHL Cb/Cc Foll). However, the other case was a high grade lymphoma (Case K - LCAL), the morphological appearance of which suggested a high malignant potential.

However, a second biopsy taken a month later from the same patient (Case L) did give rise to SCID tumours derived from the original biopsy and not simply EBV-driven lymphoproliferations. Perhaps the second biopsy was more malignant than the previous one, although it was clear that in one of the Case L-derived SCID tumours (L4), the dominant clone was that of an EBV-transformed normal B cell clone, shown clearly in the genotypic data and the Ig secretion results.

The only previous study using the SCID mouse as an animal model for NHL was carried out during this thesis. Itoh et al (1993) developed SCID tumours from 23/50 NHL specimens. Of these 23, 13 were found to originate from EBV-infected bystander B cells. Therefore the proliferation of EBV-



transformed B cells can be a problem in the growth and maintenance of these NHL-derived SCID tumours.

2 of the SCID tumours in the Itoh study resembled SCID tumour L4 in that the original NHL clone and a newly developed EBV positive B cell clone co-existed. Like this thesis, Itoh et al found that high grade lymphomas were more easily transplanted. SCID tumours from 9/11 cases in the Itoh et al study were found to be derived from the original NHL clone.

In this thesis, the SCID tumours derived from Cases F, G, H, I and 4 of the SCID tumours from Case L (L1, L2, L3, L5) were shown to be derived from the malignant clone in the original biopsies. In Cases F, G, H and I, the Epstein-Barr virus was not involved in the original biopsies or the SCID tumours. These SCID tumours retained the morphology, phenotype and Ig expression of the original biopsies. Indeed, genotypic analysis showed in the cases of SCID tumours F1-14 and G1 and suggested in the case of SCID tumour I1 that these contained identical clonal IgH gene rearrangements to that seen in the equivalent original biopsy. Unfortunately, this could not be carried out on SCID tumours H1. Further analysis showed that these SCID tumours did not share the characteristics of Ig secretion and normal diploid DNA content of the EBV (EBER/LMP/EBNA-2) positive SCID tumours. Therefore, in Cases F, G, H and I, the SCID tumours can be regarded as representative of the original biopsies and as relevant *in vivo* models for these NHL.

Case L (LCAL) displayed a restricted latency I EBV phenotype expressing EBER only, not LMP or EBNA-2 (EBNA-1 was not investigated but it is assumed that it would be expressed). In 4 of the resultant SCID tumours, this EBV phenotype was retained along with morphology, restricted antigenic phenotype and Ig expression. Indeed, genotypic analysis demonstrated an identical clonal IgH gene rearrangement, and EBV terminal

repeat analysis demonstrated that the malignant EBV positive clone in the original biopsy was present in the SCID tumours. Further analysis also demonstrated that these EBER positive SCID tumours did not exhibit characteristics of the EBV (EBER/LMP/EBNA-2) positive SCID tumours in that they showed no Ig secretion and displayed DNA aneuploidy. Therefore, SCID tumours L1, L2, L3 and L5 can be regarded to represent the original biopsy and serve as an *in vivo* model for B cell LCAL. This is the first study to show such a model for B cell LCAL.

Again, Itoh et al achieved a much better passage rate than that seen in this thesis, however additional problems such as inadequate tumour tissue for passage and infection killing the 'passage' mice were encountered in this thesis.

This study has demonstrated that NHL can be successfully transplanted into the SCID mouse. This model can be used to study the cell and molecular biology of NHL. Oncogene expression has been studied using these NHL-derived SCID tumours in our lab (O'Grady et al 1993). It was demonstrated that the SCID tumours known to represent the original NHL i.e. Cases F, G, H, I and L-derived SCID tumours showed similar patterns of oncogene expression (bcl-2, c-myc, p53) as that in the original NHL biopsies. Case F-derived SCID tumours showed relatively constant expression of the oncogenes throughout the passages. Therefore, these results suggest that these SCID tumours may provide a useful *in vivo* model for the study of aberrant oncogene expression in lymphoma.

This is the first study to provide an *in vivo* model for LCAL in SCID mice. The B cell type grown here is quite rare and therefore most studies performed on LCAL include only the T cell type. Therefore this should be a particularly useful model to study the B cell LCAL. Many comparisons have

been made between HD and LCAL and this animal model should provide an insight into the biology of B cell LCAL for comparison with HD.

Case F -derived SCID tumours passaged very well and offer a model to study tumour progression. Although a progression in oncogene expression was not seen in this case (O'Grady et al 1993), perhaps 4 passages was not adequate to investigate tumour progression. However most importantly, this SCID tumour was shown to survive liquid nitrogen and grew again retaining the phenotype (genotype was not investigated) of the previous Case F-derived SCID tumours. Therefore this SCID tumour can be used as a continuous tumour model.

New therapeutic programs are being designed for NHL i.e. monoclonal antibody therapy with attached radionucleotides or toxins or IL-2 with or without lymphokine activated killer (LAK) cells. These are being tested - their role is yet to be established (Appelbaum 1991, Longo 1991, Lim & Marcus 1992) and these SCID tumours offer a model to study this. However, only 7/25 cases of NHL (28%) resulted in SCID tumour formation, with only 5 representing the malignant clone of the original biopsies. Therefore, technical improvements are needed before this model can be used for individual NHL patients i.e. for selection of individual therapeutic protocols.

This study has shown that the SCID mouse is not a satisfactory model to study Hodgkin's disease. This was shown in this thesis by full characterisation of these SCID tumours, this demonstrating the properties exhibited by EBV-transformed normal B cells.

Transplantation of non-Hodgkin's lymphoma in SCID mice was successful in this thesis - this resulted in tumours which retained the morphology, phenotype and genotype of the original biopsies, and as such this study showed that the SCID mouse represents a satisfactory *in vivo* model to study the cellular and molecular biology of NHL.

**Appendix A    Pre-hybridisation Buffer Protocol**

**Pre-hybridisation Buffer**

<b><u>Stock</u></b>	<b><u>Volume</u></b>	<b><u>Working Dilution</u></b>
4M NaCl	150µl	0.6M
50% dextran sulphate	200µl	10%
5% sodium pyrophosphate	20µl	0.1%
10% polyvinylpyrrolidone	20µl	0.2%
10% ficoll	20µl	0.2%
0.2M EDTA (di-sodium salt)	25µl	5mM
1M tris pH 7.5	50µl	50mM
10mg/ml DNA sheared salmon sperm boiled (10 mins) and cooled before adding	15µl	150µg/ml
100% formamide	500µl	50%
<b>Final Volume</b>	<b>1000µl</b>	



## **Appendix B    Alkaline Phosphatase Substrate for Visualisation of *in situ* Hybridisation**

### **0.1M Tris Buffer pH9.5 for BCIP/NBT Substrate**

0.1M Tris	6.050g
0.01M NaCl	0.292g
0.005M MgCl <sub>2</sub> (50mM)	0.238g

Dissolve in 450ml of distilled water. Adjust pH to 9.5, make up to 500ml with distilled water.

### **Substrate**

To 50ml of 0.1M Tris pH9.5, add 1ml of 50mM levamisole.

NBT 75mg/ml in 70% dimethylformamide	198 $\mu$ l
BCIP 50mg/ml in 70% dimethylformamide	150 $\mu$ l
0.1M Tris pH9.5 + levamisole	45ml

## **Appendix C    Molecular Analyses**

All the molecular analyses performed on the original lymphoma biopsies and the SCID tumours were carried out by Alice Gallagher in the Department of Veterinary Pathology in the University of Glasgow Veterinary School.

DNA was extracted from samples using a standard Proteinase K and Sodium Dodecyl Sulfate digestion followed by phenol and chloroform extractions to remove protein. Samples were resuspended in Tris EDTA.

### **PCR For IgH Gene Rearrangements**

DNA samples from frozen biopsies and frozen SCID tumour blocks (Section 2.4) were examined for the presence of IgH gene rearrangements using a PCR strategy. The primers used were common variable and joining region primers homologous to the V and J regions of the IgH gene locus situated on chromosome 14.

The following primer sequences were used : (bases in parentheses show alternative bases at 2 positions)

primer 1(V)	5' CTG TCG ACA CGG TGT ATT ACT G 3'
primer 2(J1-J5)	5' CTC(T) ACC TGA G(A)GA GAC GGT GAC C 3'
primer 3(J6)	5' CAA AGG CCC TAG AGT GGC CAT T 3'

One microgram of DNA was amplified in a 50µl reaction volume using 1 unit of Taq polymerase, 50 pmols of each primer, 200µM/L of each deoxynucleoside triphosphate and 1 x buffer containing 10mM Tris pH 8.2,

50mM KCl, 1.5mM MgCl<sub>2</sub> and 100µg/ml gelatin. A negative control of water was included for every 2 samples analysed. A positive control sample from a B cell Lymphoma was included in each assay.

40 cycles of amplification were performed in a programmable heat block after initial denaturation for 7 minutes at 95<sup>0</sup>c. Each cycle included ramping to 94<sup>0</sup>c for 1 minute, at temperature for 10 seconds, 2 minutes to reach 55<sup>0</sup>c, at temperature for 10 seconds, and finally ramping to 72<sup>0</sup>c over 1 minute and at temperature for 30 seconds.

10µl of the products of the PCR experiments were run on a 8% polyacrylamide gel and visualised on a UV transilluminator after staining in 500µg/ml ethidium bromide for 10 minutes.

### **Southern Blot Analyses for Detection of EBV**

Southern blot analysis was performed to investigate for the presence of the Epstein Barr Virus. The BamW probe which detects the BamW repeats of the virus was used to determine the EBV status. The EcoR1D probe, which detects the terminal repeats of the virus was used to determine clonality of the samples containing EBV.

10µg of DNA were digested with BamH1 enzyme or BamH1 and EcoR1D double digest for hybridisation to the BamW and EcoR1D probes respectively. Digests were run on 0.8% agarose gels for 16-18 hours at 32 volts. DNA was transferred to nylon membrane after initial denaturation and neutralisation of the gels using a standard capillary action southern blot technique. Nylon filters were stratalinked to bind DNA to the membrane for 0.8 minutes at 1200 joules.

50ng of BamW and EcoR1D probes were labelled using the Multiprime labelling system and  $^{32}\text{P}$  labelled alpha dCTP (3.0 MBq of 800 Ci/mMol).

Filters were pre-hybridised in 2.5 mls of 50% formamide southern buffer for 4 hours and after addition of labelled probe (1 million counts/ml) were hybridised for 16-20 hours. Washing to remove excess unbound probe was carried out at room temperature for 10 minutes in 2x SSC/0.1% SDS, followed by 2x 1 hour washes in 0.5x SSC/0.1% SDS. Excess moisture was removed from the filters which were then transferred to an autoradiograph cassette with film and exposed for 1 day initially, followed by a 7-10 day exposure.

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